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THE PATHOLOGICAL EFFECTS OF ATMOSPHERES RICH IN OXYGEN.

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Plates 20 to 23.

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Studies of the pathological effects of oxygen administered under various conditions have not been so numerous or exhaustive as the investigations of its chemical and physiological relations. The fact that oxygen is being used freely as a therapeutic agent, as a prophylaxis against asphyxia in anesthesia, as a protective agent in certain industries, in some forms of deep sea diving apparatus, and in high ascents into the air, makes the study one of practical importance. Even today there is much difference of opinion as to how far oxygen plays a part in the production of some of the manifestations of caisson-disease. Furthermore, oxygen is used extensively in respiration chambers, such as those of the Carnegie Nutrition Laboratory, but the percentage of oxygen in the atmosphere of the chamber is rarely that of out-door air; *i.e.*, 21 per cent, yet it is rarely below 18 or above 24 per cent. The present study deals with the effects on the organism of high oxygen partial pressures (80 to 96 per cent) under ordinary barometric pressure, particularly from the point of view of pathological anatomy and histology. The brief extracts from the literature are taken from the papers that bear directly on the problem in hand.

According to Schmiedehausen (1), Lavoisier shortly after his discovery of oxygen in the atmosphere demonstrated that pure oxygen produces congestion and even inflammation of the lungs. With the introduction of ether and chloroform, the use of oxygen to combat asphyxia began and in later times deep sea diving and the use of oxygen in various industries turned the attention of physiologists and pathologists to studies of the effects of oxygen inhalation. Paul Bert

(2) emphasized the effects on the nervous system of high atmospheric pressures, but apparently overlooked the effects on the lungs. In contrast to this, Lorraine Smith (3) pointed out the inflammatory reaction in the lungs of mice and also called attention to the appearance of moderate congestion in the abdominal viscera. Smith claims, however, that fibrin and leukocytes play no part in inflammation. He found that 40 per cent oxygen for 8 days did not produce pneumonia, but that 80 per cent killed 2 mice in 2 days, while 2 others survived unharmed. An average pressure of 125.3 per cent oxygen killed mice in an average of 64 hours; 180 per cent oxygen killed in 24 hours; and 300 per cent oxygen produced pneumonia in 5 hours. Heller, Mager, and von Schrötter (4), in their extensive studies, ignore pneumonia as a factor in caisson-disease, but Hill and Macleod (5) confirm the findings of Lorraine Smith in regard to pneumonia; they agree, however, with Paul Bert and von Schrötter that in caisson-disease the liberation of compressed gases in the tissues is the most important pathological effect. Hill and Macleod call attention to a considerable variation in individual resistance to oxygen poisoning, "but the larger animals seem just as susceptible as mice." They also state that in regard to production of pneumonia their animals were somewhat more resistant than those of Lorraine Smith.

Schmiedehausen (6) in a series of experiments with 13 animals (2 mice, 3 rabbits, 8 guinea pigs) used pure oxygen by tracheal cannula in 2 animals and high percentages of oxygen in chambers for the rest. His pathological findings are not critically described, but he finds in a general way hyperemia, more or less marked atelectasis, focalized edema, and inflammatory processes. He lays much stress on minor changes after short exposures, but demonstrates no true pneumonia in less than 69 hours' exposure. Schmidt and David (7) caution against the too free or prolonged use of oxygen in chloroform anesthesia. They state that percentages of oxygen as low as 40 to 60 may produce inflammatory changes in the lungs after 70 hours' exposure. David (8) confirms the work of Schmiedehausen and suggests the use of high oxygen partial pressures for the purpose of inducing a pulmonary hyperemia for therapeutic purposes. Bornstein and Stroink (9), working principally with rats under increased barometric pressure, in order to determine the advisability of the use of oxygen by divers, state that high pressures (5 atmospheres of oxygen) for periods as short as 2 to 4 hours produce alveolar edema, desquamation, and slight hemorrhage. They call attention to the fact that the lung findings are similar to those of slightly irritating gases, such as ether, and state that in their animals no organs except the lungs show changes. They state that lower pressures of one to three atmospheres for 20 to 48 minutes are harmless. Schmidt and David (10) regard Bornstein and Stroink's work as confirmatory of their own.

The purpose of the present study is to examine the thoracic and abdominal viscera, as well as the hematopoietic system, in an effort

to explain the more obvious results in the lungs. There is included also a critical study of the pathological histology of the lung changes in comparison with the lungs of control animals. Throughout the study the most careful attention has been given controls, instead of placing any great reliance on the theoretical condition called normal. As will be seen from the reports on different organs, this precaution, particularly in studies on the rabbit, is of extreme importance.

For the purpose of the study the animals were placed in the small animal apparatus devised especially for this investigation by Benedict (11). A supply of carrots sufficient for three days was placed in the chamber and replenished in those cases where the experiments were considerably prolonged. No animal at autopsy showed an empty stomach. Water was supplied freely at all times. Brünig (12) claims that the effects reported by Bornstein and Stroink (13) are due to excessive dryness of the atmosphere rather than its rich oxygen content,[1] but the Benedict apparatus provides for moistening the air.

As the study progressed the details of the examinations varied somewhat. In a few of the earlier experiments animals from stock were placed in the chamber and autopsies performed as soon after death as possible, or the animals were removed from the chamber on exhibition of marked dyspnea, killed by a blow, and the autopsy was performed. It was soon considered advisable to examine the urine before exposure in the chamber and to exclude from the study all animals with albuminuria. The examinations increased in complexity, however. Each animal before entering the chamber was examined for albuminuria, complete blood counts were made, clotting time was determined, hemoglobin estimated, and the percentage of reticulated erythrocytes estimated. The animals that were removed alive from the chamber were examined in the same way, and in addition a sufficient amount of blood was removed from the femoral artery to permit determination of the erythrocyte resistance to hypotonic salt solutions. In spite of the fact that frequently examinations for

[1] Bornstein later wrote to the *Deutsch. med. Wchnschr.* (1912, xxxviii, 2035) maintaining that his controls were sufficient to overcome the objection made by Brünig, to the effect that dryness of the atmosphere in the oxygen chamber produced the pneumonia.

albumin were made on two successive days preceding the exposure to high oxygen, the autopsy on several occasions showed chronic nephritis. This matter will be discussed later.

Twenty-one of the 55 rabbits studied were control animals. Seven of these were in room air in a cage the same size as the special chamber and under the same conditions as to food as those in the chamber. The other controls were in the chamber, with oxygen kept at a low percentage; 6 animals at 21 per cent, 2 at 23 per cent, 1 at 27 per cent, 2 at 28 per cent, and 2 at 31 per cent. One control animal was taken directly from stock.

The pathological examination includes gross and microscopical examination of the heart, lungs, liver, kidney, adrenal, spleen, lymph nodes, and bone marrow, as well as gross examinations of the aorta, stomach, and intestines. Blocks were placed in Zenker fluid and in 10 per cent formalin solution. The routine stain was hemalum and eosin, and in addition the lung sections were stained with the Mallory connective tissue stain for fibrin; numerous frozen sections of the heart, kidney, and liver were stained with Scharlach R for fat.

The pathological findings were at first grouped according to finer grades of difference in oxygen percentage; namely, 70 to 75 per cent, 76 to 80 per cent, 81 to 85 per cent, etc. It was found, however, that these minor changes in percentage were not of apparent importance and that a grouping of 60 to 80 per cent and 80 per cent and higher gave results that were easily distinguishable. The following description of organs is confined to animals which had been exposed for varying periods of time to atmospheres containing 80 per cent and more oxygen. Further studies of lower percentages are now in progress. As will be seen, the examination of these organs is constantly compared with that of the control animals.

Heart.

The examination of the hearts of 19 control animals showed numerous departures from the normal, particularly in the presence of fat in the muscle, foci of chronic interstitial myocarditis, chronic fibrous pericarditis, and in 2 of the 19 cases slight dilatation.

Hearts of Animals in High Oxygen Partial Pressure.—The results of the examination of 26 hearts in this series can be condensed into

the following statements, with the reservation that the changes noted in the control hearts were also found in the oxygen hearts in practically the same proportions.

First, of 9 animals that died in the oxygen chamber the hearts of 7 showed notable dilatation either of the right or of both sides. Two other animals that survived respectively 2 and 5 days' exposure to high oxygen atmospheres also showed bilateral cardiac dilatation. Second, as exposure to high oxygen becomes prolonged, cloudy swelling, meaning loss of transverse striations and coarse granulation in the protoplasm of the fiber cell, becomes practically uniform. Several cases of acute non-suppurative interstitial myocarditis are found in the hearts of high oxygen animals, but these are not sufficiently frequent to be of significance. Of 16 examinations for fat in the high oxygen animals 14 resulted positively as compared with 7 in 11 normal animals,—a difference probably within the margin of error of biological experimentation, but possibly associated with the practically constant cloudy swelling noted above. Litten, Naunyn, Nasaroff, Werhovsky, and Welch have shown that exposure of animals for several days to a temperature of 40° C. results in fatty degeneration of the heart muscle (14). In none of our experiments did the environmental temperature rise above 25° C.

Aorta.

The aortas of 24 animals were examined grossly and arteriosclerosis was found in 3 cases, all of which were controls. It can therefore be stated that no notable effect is produced in the aorta.

Lungs.

Grossly the lungs of the so called normal animals have the normal color and consistence, and crepitate throughout. Occasionally areas of slight congestion are observed posteriorly at the base of one or both lungs (3 animals). One animal showed marked distention of the lung, and a few minute subpleural hemorrhages.

Microscopically the picture with three exceptions is practically constant. The larger blood vessels exhibit wide open lumina containing blood corpuscles; in some of the smaller vessels the lumina

contain fine acidophilic granules, probably the precipitated protein of the serum. The bronchi are of normal size and contain a small amount of granular precipitate, which does not stain for fibrin, but which immediately overlies the epithelium. The epithelium shows well marked granulation of the protoplasm and in many cases contains mucin. Desquamation of epithelium is frequently seen and occasionally there occur small mononuclear cells, probably lymphocytes, in the lumina of the bronchi. In small foci the alveoli are distended and the walls thin, but the lungs for the most part show well marked capillary congestion, even to a point where the capillaries appear as small bullæ projecting into the alveolar spaces. Leukocytes are numerous in the capillaries and in some cases form a striking part of the picture; they sometimes appear within the alveoli. The alveolar epithelium is normal except that occasional desquamated cells showing cloudy swelling are seen. Alveolar edema cannot be demonstrated. In three animals the alveoli are seen to be markedly distended, but the bronchi remain the same as in the other lungs. Throughout the series small lymph nodes are found in relation to the bronchi, made up principally of small lymphocytes with a few large mononuclears included.

Lungs of Animals in High Oxygen Partial Pressure for Approximately Twenty-Four Hours.—These 6 lung specimens show little material departure from the normal except that congestion is slightly more marked (Fig. 1, Rabbit 10). Leukocytes are apparently not more numerous than in many normal lungs. Cloudy swelling and desquamation of alveolar epithelium are seen in all these lungs, and in one lung (Rabbit 13) there is a small area of consolidation made up largely of desquamated alveolar epithelium, which has fused in places to form giant cells. The bronchi show no material changes, but the lymph nodes are enlarged and contain many large mononuclear cells.

High Oxygen for Approximately Forty-Eight Hours.—Of the 9 lungs studied in this series, 5 (Nos. 19, 29, 30, 44, 54) show no more change than do the lungs of the preceding group. Rabbit 18 shows in addition a small focus of desquamative bronchopneumonia similar to that seen in Rabbit 13, but showing more fibrin and leukocytes. In Rabbits 20 and 21 alveolar and bronchial desquamation are marked and numerous alveoli contain a small amount of fine granular acidophilic precipitate in addition to a moderate deposit of fibrin strands. In Rabbit 4, which died in the oxygen chamber, the edema and fibrin are richer, occasional alveoli being almost filled with fibrin. Numerous polymorphonuclear leukocytes appear in the alveoli. As has been already indicated, these differences cannot be accounted for by minor differences in oxygen percentage.

High Oxygen for Approximately Three Days.—Of the 6 animals forming this series the lungs of all save one show serious alterations. In Rabbit 23 intense congestion is present, but the changes are not more marked than those pictured in Fig. 1. All the others show somewhat varying degrees of edema, epithelial desquamation, exudate of leukocytes, and fibrin formation,—in other words, varying degrees of pneumonia. Of the 5 lungs showing this inflammation only one shows complete filling of the alveoli, and in this case the edema occupies most of the space. The bronchi show cloudy swelling of the epithelium, but little mucin formation; there is infiltration of polymorphonuclear leukocytes into the wall and into the lumen; there is marked epithelial desquamation and some of the bronchi show fibrin formation and an occasional erythrocyte. The peribronchial and perivascular lymphatics are frequently the seat of edema. The blood vessels of all kinds and sizes are considerably distended. The alterations in the alveoli are shown in Figs. 2, 3, and 4. They consist of cloudy swelling, and occasionally fatty degeneration of the attached epithelium, desquamation, advanced cloudy swelling, and necrosis; there is moderate infiltration of polymorphonuclear leukocytes and lymphocytes; edema appears as a fine acidophilic precipitate, occupying usually only small areas in the alveoli, but in one case (Rabbit 49) filling the alveolar spaces; fibrils, nodules, and networks of fibrin appear usually in irregularly disposed foci, occupying sometimes a small part, sometimes all of an alveolar space. The fibrin has the typical histology, is acidophilic, and takes the red stain by the Mallory connective tissue method.

High Oxygen for Approximately Four Days.—Of the 4 animals comprising this series one (Rabbit 56) shows only slight changes in the lungs, two (Rabbits 1 and 2) show exudation as seen in most of the lungs of the preceding series, and one (Rabbit 5) shows marked edema in addition to the inflammatory exudate and is similar to the lung of Rabbit 49 of the preceding series. It can safely be said that exposures of approximately 4 days produce practically the same alterations in the lungs as are seen at the end of 3 days.

Rabbit 6 survived 4 days and 20 hours in 83 per cent oxygen. Grossly the lungs were mottled with small areas of congestion. Histologically the lungs show the same marked congestion, epithelial desquamation and degeneration, leukocyte infiltration, moderate edema, and fibrin formation observed in lungs of shorter exposure to high oxygen atmospheres. Two notable additional changes are observed; namely, slight clumping of fibrin in a few alveoli and in a few instances perivascular edema with leukocyte infiltration.

Rabbit 3 survived 7 days' exposure to an atmosphere of 82 per cent oxygen. Grossly the lungs showed mottled areas of congestion and well marked edema. Histologically the lungs show the same changes as Rabbit 6, including the clumping of fibrin and the perivascular edema. The alveolar edema is somewhat more marked than in the preceding animal.

To summarize the examination of the lungs it can be said that exposure for 24 hours to atmospheres containing 80 per cent and

more oxygen produces little material change in the lungs. Exposure of approximately 48 hours may show nothing of moment or may lead to the development of marked congestion, edema, and early fibrinous bronchopneumonia. Exposure of approximately 3 days leads almost constantly to fibrinous bronchopneumonia which shows little change after 5 or 7 days.

The inflammatory process in the bronchi, the marked desquamation of alveolar epithelium, associated with fibrinous exudation into the alveoli, establishes the diagnosis of fibrinous bronchopneumonia. At no stage that we have observed does there appear the rich infiltration of leukocytes seen in true croupous or lobar pneumonia or in the advanced bronchopneumonia of man.

Grossly the pneumonic lungs showed an extremely variable distribution of the consolidated areas. Sometimes the process was diffuse, but even here the sharp edges of the lung frequently escaped the process; more commonly the process was irregularly distributed in small foci, 4 or 5 to the lobe. The color of the pneumonic areas was usually a deep red or bluish red, but a few areas were reddish gray. The cut surface was constantly moist and from it could be expressed frothy, salmon colored, limpid fluid. Sometimes small blocks sank in water, and others floated.

Liver.

The livers of 19 control animals were examined; 7 are absolutely normal, and 7 more show such slight changes as to be regarded as practically normal. The pathological alterations in the controls include perilobular fibrosis, coccidiosis, focal necrosis, hemosiderosis, hydropic degeneration, and in one case distinct central congestion.

Livers of Animals in High Oxygen Partial Pressures.—These organs also show the changes enumerated above in several instances, but the general picture can be presented in the statement that the principal change in the livers of animals exposed to high oxygen atmospheres is passive congestion, which may or may not be associated with hemosiderin pigmentation. This change appears in 24 hours, but is not marked until about 72 hours have elapsed, and thereafter is associated with intercellular edema, indicated by a deposit of albuminous granules between liver cells and between cell columns and sinusoidal endothelium.

Stomach and Intestine.

The gross examination of these organs showed the presence of food in all stomachs. Congestion was noted in 4 of the oxygen animals and in none of the controls. Submucous hemorrhages were found in the stomachs of 4 oxygen animals and in one control. An additional oxygen animal showed submucous hemorrhages in the upper ileum.

The gastro-intestinal tract, therefore, takes part in the general congestion found in the animals exposed to the high oxygen atmospheres.

Kidneys.

The histological examination of the kidneys of the control animals shows normal glomeruli except that in 8 of the 19 cases the loops of the glomerular tuft are unusually well filled with erythrocytes. In 4 kidneys congestion is more extensive. In 11 kidneys examined for fat, 9 show it in the loops of Henle, and in 4 of these there is additional involvement of the convoluted tubules. In 2 kidneys no fat can be demonstrated microscopically. Seven kidneys show chronic interstitial nephritis, and 4 show foci of lymphoid and plasma cells in the interstitial tissues, these changes being combined in two cases (15). Sixteen animals were examined for albuminuria before being used as controls and also after they had been in the open cage or in the chamber with practically normal oxygen content at the Nutrition Laboratory, and 5 of these on later examination showed albuminuria. Histologically, the kidneys from these 5 show chronic interstitial nephritis. Two kidneys showing chronic interstitial and one showing subacute interstitial nephritis excreted urine which was negative for albumin by the tests employed.

Kidneys of Animals in High Oxygen Partial Pressure for Approximately Twenty-Four Hours.—Six kidneys were studied in this series, and 5 show well marked general congestion. All show glomerular tufts well filled with blood. Of 3 animals examined for albuminuria, 1 was positive, the same animal being the only one of the 6 which shows a well marked chronic interstitial nephritis. Two kidneys (1 of them from the case of chronic interstitial nephritis) show albuminous degeneration to a slightly more marked degree than was found in the controls. Five were stained for fat, 4 show fat in the loops of Henle, and in 1 of these the fat appears throughout the cortex.

High Oxygen for Approximately Forty-Eight Hours.—Of the 9 kidneys in this series all but 1 show moderate or marked congestion. The subcapsular spaces of one animal show albuminous precipitate. Cloudy swelling of the epithelium of the convoluted tubules is present in all but 2 specimens. Eight specimens were examined for fat, 7 show fat in the loops of Henle, and in 2 of these the fat is found also in the convoluted tubules. The kidney of 1 rabbit shows hyalin casts in the tubules, but there is a coincident chronic interstitial nephritis. Three kidneys in this series show chronic interstitial nephritis, and 3 show subacute interstitial nephritis, the two being combined in two instances, thus making a total of 4 kidneys showing interstitial changes in this series. Albuminuria appeared in 6 of 7 examinations, in 4 instances associated with interstitial changes, in 2 cases with no marked change other than congestion.

High Oxygen for Approximately Three Days.—In this series the glomeruli are normal except for well marked congestion. The convoluted tubules show slightly more cloudy swelling than do those of the control animals. Two kidneys show fat in the epithelium of the loops of Henle. Very slight chronic interstitial nephritis is present in 3 kidneys and marked chronic interstitial nephritis in 1 other kidney. General congestion is marked in 5 kidneys and moderate in 1. Three animals had no urine in the bladder on return from the oxygen chamber. Of the other 3 only 1 showed albuminuria, and its kidney shows very slight chronic interstitial nephritis.

High Oxygen for Approximately Four Days.—Three kidneys of this series were examined histologically and all show marked filling of the tuft capillaries by blood. In 2 of these, albuminous granules are present in the subcapsular space; all 3, however, are the seat of chronic interstitial nephritis, and of these, 2 are also the subjects of subacute interstitial nephritis. Cloudy swelling is distinct in all 3, and 2 examined for fat show it in the loops of Henle. Congestion is marked in all. The one animal with urine in its bladder after return from the oxygen chamber showed no albuminuria.

The kidneys of the animals that survived 4 days', 20 hours', and 7 days' exposure show no additional changes. Congestion is marked in both and the connective tissue is normal in both. The 7 day animal shows fat in the loops of Henle; the other was not examined for fat, and neither had any urine examination.

The important change in the kidney is the appearance of congestion. Cloudy swelling appears to be more distinct in the kidneys of the experimental animals than of the controls, but this is such a fine change that its importance can easily be overestimated, particularly when the controls show minor degrees of the same process, evidently as the result of the histological technique employed. Albuminuria appeared in 8 cases, and in 6 of these the corresponding kidneys show some degree of interstitial change. Two animals showed albuminuria

after exposure of 48 hours to high oxygen. Their kidneys histologically are free from interstitial change and the greatest probability in so far as the kidney is concerned is that the albuminuria was probably due to passive congestion. Seven negative examinations following exposures of 24 hours or more would indicate that the high oxygen atmospheres, if responsible at all for albuminuria, produce it only occasionally, provided the kidney is normal at the start of the experiment, and then probably as the result of passive congestion. The 6 positive cases of interstitial nephritis are probably to be explained by the superaddition of passive congestion to a kidney already diseased, but diseased so that under ordinary conditions albuminuria did not exist.

Adrenals.

The adrenals of 16 controls were examined, and those of 14 were found to be plump organs well filled with lipoid material. Two were small with almost solid cells very poor in lipoids.

The adrenals of 16 oxygen animals show no changes sufficient to justify their grouping into sequential series. Six of the 16 show congestion in somewhat more marked degree than the controls, and 1 of these shows a small area of hemorrhage into the middle of the cortex. Fourteen show rich lipoid content and 2 do not. Two show focal necrosis, 1 in the same degree as exhibited in a control animal, and 1 shows somewhat larger and more frequent foci. This cannot be ascribed to postmortem change, as the control and the 2 oxygen animals were received alive and the organs placed in fixatives immediately after killing; furthermore, the reaction is characteristic of that taking place in living tissues.

It is therefore to be concluded that the adrenals show no change other than congestion as the result of exposure of the animals to high partial pressures of oxygen.

Spleen.

Comparatively little variation is found in the spleens of the controls except that some are more richly pigmented than others, the connective tissue content differs slightly, and the germinal centers of some spleens show more active mitosis than others.

Spleens of Animals in High Oxygen Partial Pressures.—Twenty-three spleens were examined in this series and 16 show well marked congestion. Moderate hyperplasia of endothelial cells in the pulp and sinuses is a frequent finding, but it is not more frequent than in the controls. Phagocytosis of pigment granules and of nuclear fragments is found in both controls and oxygen animals. Eleven of the 23 spleens show a few endothelial cells containing a single or only a few erythrocytes. This change is found in 2 of the control spleens; the same 2 spleens are the only controls to show congestion, and in both series this change does not occur independently of congestion. With the exception of a rabbit which was in the chamber for 2 days and 22 hours, whose spleen shows very active phagocytosis of erythrocytes, the degree of phagocytosis does not appear to be any greater in the oxygen spleens than in the controls. According to the same arguments that will be advanced in consideration of the changes in the lymph nodes and also in the general discussion, the phagocytosis of erythrocytes is regarded as part of the phenomena of congestion rather than specifically the result of the high oxygen exposure. It is therefore concluded that no specific alterations occur in the spleen as a result of the high oxygen exposure other than congestion and its incident changes.

Lymph Nodes.

Attention was not directed to the lymph nodes until after the experiments were well under way. Consequently, the number studied is smaller than is the case with other organs. Fifteen controls show large active germinal centers in all but 1 case. The sinuses show large numbers of endothelial cells, and in 10 of the nodes active mitosis is to be seen in many of these cells. In 8 of the nodes amphophilic leukocytes are to be seen in considerable numbers. Pigment is constantly present and in 4 instances large amounts are found; it occurs principally within large mononuclear and multinuclear cells of the sinuses and to a lesser degree in the large mononuclears and occasional multinuclears in the follicles. In 1 lymph node there is marked phagocytosis of erythrocytes on the part of the endothelial cells of the central sinuses. Five other lymph nodes show after careful search an occasional endothelial cell in which a few erythrocytes have been englobed.

Lymph Nodes of Animals in High Oxygen Partial Pressure for Varying Periods of Time.—Of animals exposed to high oxygen for from 24 to 72 hours 9 groups of lymph nodes were examined. Of these all show the same large proliferating germinal centers as the controls except 2 (Rabbits 24 and 49), but as these animals died in the chamber the appearance of necrosis in the germinal centers and in many of the cells in the sinuses leads one to suspect that this is largely due to postmortem change. All the nodes show considerable amounts of pigment and 5 show markedly large amounts of pigment, distributed in all cases as in the controls. Six cases show phagocytosis of erythrocytes on the part of sinus endothelium, and in 3 of these the process is marked. The most marked case of pigmentation fails to show such phagocytosis.

It would appear, from the material available, that following exposure to high oxygen atmosphere there is somewhat more marked phagocytosis of erythrocytes in the lymph nodes. Three of these cases are associated with pneumonia and 3 are not; 3 are associated with notable cardiac dilatation and 3 are not. All are associated with general congestion as observed in the kidney and spleen, and 3 are associated with similar phagocytosis in the spleen. The process appears to be more clearly associated with passive congestion in other organs than with any other demonstrable change.

Bone Marrow.

In examining histologically the bone marrow of 15 control rabbits, variations are noticeable, particularly in the proportionate amounts of fat and functionating cellular tissue. Eight of the 15 show in the fat spaces an acidophilic granular mass; in the others the fat spaces are clear. General cellular hyperplasia is found in all but 3 animals, in only 1, however, to any considerable degree. Only 1 presents a strictly normal picture, and 2 others are aplastic. One specimen shows slight general hyperplasia and also small foci of necrosis. Mitotic figures are commonly seen and fine golden brown granules of pigment, sometimes intracellular, again in extracellular position, are frequent. All but 3 specimens show a rich blood content.

The leukoblastic and erythroblastic centers, as described by Bunting (16), and the platelet formation as described by Wright (17), can be made out easily. The number of megakaryocytes appears to be extremely variable.

Specimens of marrow from 10 animals exposed to oxygen atmospheres of 80 per cent and above show no distinct departure from the pictures seen in the controls. Two are quite normal. Six show the acidophilic granular mass in the fat spaces. Hyperplasia of all the elements or of either the leukoblastic or erythroblastic centers does not exceed that of the controls. Megakaryocytes and mitotic figures are present in normal numbers. Congestion is marked in 3 specimens (Nos. 23, 24, and 49).

Erythrocyte Counts.

Erythrocyte counts were made on 12 controls before and after the experiments. The counts before experiment varied between 5,512,000 per cmm. and 8,460,000 per cmm., but were largely in the neighborhood of 6,000,000. After the experiment 7 showed increases of from 300,000 to 1,990,000 (average 1,012,000), and 4 showed decreases of 456,000 to 1,312,000 (average 768,500). One animal showed no change. The counts were made under as nearly similar conditions as possible, but these variations made it seem unlikely that the oxygen animals might show much that would be distinctive.

Five oxygen animals, the blood of which was counted before the experiment, showed variations of from 5,120,000 to 7,320,000 erythrocytes per cmm. Four animals showed an increase after exposure in the chamber of from 488,000 to 2,788,000 (average 1,313,000), and 1 showed a decrease of 1,080,000. This last animal was in the high oxygen for approximately 4 days, whereas the others varied between approximately 48 and 72 hours.

It would appear that prolonged exposure to oxygen produces no material changes in the erythrocyte count that are not observed in control animals living for similar periods under the same general conditions.

Resistance of Erythrocytes to Hypotonic Salt Solutions.

The resistance of the erythrocytes to hypotonic salt solutions was determined by the technique described in a previous communication (18). It was, of course, impossible to obtain sufficient blood for the test before the experiments, hence the control of each test was either

an animal from stock, killed for the purpose, or in several cases where 2 animals were observed simultaneously, 1 in the chamber and 1 in the room air, the latter served as a control. Of 10 control animals tested against an animal from stock, 5 showed the same corpuscular resistance, 5 showed increases in resistance corresponding to 0.050 per cent sodium chloride, and 1 showed an increase corresponding to 0.100 per cent sodium chloride.

Of 5 high oxygen animals tested against an animal from stock, 4 showed an increase of resistance equal to 0.050 per cent sodium chloride, and 1 an increase equal to 0.100 per cent sodium chloride. Of 3 animals tested with those that had been for the same time under similar conditions no change in corpuscular resistance could be demonstrated.

Reticulated Erythrocytes.

Attempts were made to determine the percentage of reticulated erythrocytes in the circulating blood, but in control animals this was found to vary between 2 and 25 per cent, a variation too wide for satisfactory work. The ground covered by this procedure is also covered by the work with resistance of the erythrocytes to hypotonic salt solutions.

Hemoglobin.

Most of the hemoglobin determinations were made with the Tallquist scale, as it was believed that to be of any importance in the rabbit, the hemoglobin difference must be large enough to show on this scale. No such differences could be demonstrated. The Dare hemoglobinometer was used with 2 controls and 2 high oxygen animals. One control showed an increase from 90 to 98 per cent, and the other a decrease from 80 to 72 per cent. Two high oxygen animals showed decreases respectively from 95 to 88 per cent and from 93 to 88 per cent, the first being in the chamber for 2 days, the second for 4 days. The hemoglobin determinations are therefore no more conclusive than the erythrocyte counts.

Leukocyte Counts.

The leukocyte count of rabbits is very variable within certain limits, and although all the counts were made at noon or early in the

afternoon (so as not to follow the late afternoon feedings), the 9 controls showed primary counts of from 5,400 to 13,200. After the experiment 4 showed increases of from 1,600 to 7,400 (average 3,950), 4 showed decreases of from 500 to 2,800 (average 2,175), and 1 remained unchanged.

Of the high oxygen animals, 1 (No. 25) showed 19,800 before and 15,100 after the experiment. No. 26, not counted before, showed 4,800 after the experiment. Of 4 others 2 showed slight increases (600 and 1,400) and 2 showed distinct increases (3,000 and 6,800) in the number of leukocytes. These may be tabulated as follows (Table I):

TABLE I.

Rabbit No.	Before experiment.	After experiment.	Increase.	Decrease.
25	19,800	15,100		4,700
26		4,800		
29	7,000	8,400	1,400	
30	8,400	11,400	3,000	
48	4,100	10,900	6,800	
56	7,400	8,000	600	

Rabbit 25 is excluded as being unsatisfactory because of the high primary count. It is safe to say, however, that Rabbit 26 shows no increase even though no primary count had been made; this animal had a distinct pneumonia. Rabbits 29 and 56 show a slight increase and were free from pneumonia. Rabbits 30 and 48 show marked increases; No. 30 had no pneumonia and No. 48 had pneumonia. It would appear that the increases are not dependent upon the presence of a pneumonia, and with the wide variations seen in control animals, variations which are not exceeded by the oxygen animals, the differences in leukocyte count are probably to be explained only as accidental variations so frequently seen in the rabbit.

Clotting Time.

Clotting time was determined by the Brodie-Russel Boggs coagulometer. Thirteen controls and 5 high oxygen animals failed to show any material differences in the clotting time as observed before and after the experimental periods.

DISCUSSION.

A discussion of the results of this work cannot be complete without calling attention to the appearance in rabbits, with a fairly high degree of frequence, of lesions not the result of experimental procedures, the so called spontaneous lesions. Without the study of numerous controls the present study might have led to false conclusions, notably in the matter of fatty degeneration of the heart. Fortunately, most of the non-experimental lesions are of subacute and chronic nature and could not reasonably be attributed to the relatively short exposure in the chamber. There is in rabbits a distinct individual variation in resistance to oxygen, as pointed out by Hill and Macleod (19), and also shown in the present series; these variations might well be accentuated by living through the diseases leading to these non-experimental lesions. There is, however, no final and conclusive evidence to support this view. The chances of error in biological experiments include this individual variation and can only be overcome by the use of a considerable number of animals.

The accuracy of study of the changes in the lungs is favored by the fact that non-experimental lesions of the lungs are rare, but on the other hand individual variations in the development of the pneumonia are fairly well marked and in the study of the lesions in this series of experiments are favored by the number of animals included. As has been shown, the lesion is a fibrinous bronchopneumonia in which edema and desquamation play a prominent part. Lorraine Smith (20) stated that fibrin plays no part in the inflammation, but our studies controvert this statement in so far as the rabbit is concerned. His statement in regard to the unimportance of leukocytes in the process is borne out in our work. The resemblance of this pneumonia to that produced by irritant gases has been noted by Bornstein and Stroink (21), and the resemblance to the earlier forms of pneumonia following nitric oxide poisoning is also to be mentioned. The absence of any well marked leukocytic infiltration in the pneumonic area and the absence of demonstrable leukocytosis in the circulating blood point toward a pneumonia of irritative rather than of bacterial origin. The lesion in oxygen poisoning develops in from two to three days and the individual variation is such that the state-

ment of Schmiedehausen and others, that the pneumonia appears in a certain number of hours, cannot be supported. The German workers particularly call attention to changes appearing in a few hours, but when compared carefully with controls the significance of these minor changes has certainly been overestimated and the percentages of oxygen used in the present series cannot be said to have produced any important changes in less than 24 hours; and exposures of approximately 24 hours have not in our experience produced any notable change other than congestion. The fact that numerous animals showed dilatation of either the right side or both sides of the heart must be considered in reference to the lung changes and the appearance of congestion of the abdominal viscera.

Studies of the influence of oxygen inhalation on circulation have not been numerous. Benedict and Higgins (22) have demonstrated a slowing of the pulse following the breathing of atmospheres containing 40, 60, and 90 per cent oxygen, and the work has been confirmed by Parkinson (23), but no studies were made directly of the pulmonary circulation. Retzlaff (24), by plethysmographic studies, has shown that oxygen produces vasoconstriction in the lungs, and he therefore argues improved pulmonary circulation. He does not show how long the vasoconstriction may persist and hence gives no conclusive evidence as to the effect of prolonged oxygen inhalation on the right ventricle.

If vasoconstriction were prolonged, it is conceivable that the right heart might fail under the demand for increased work, and in this event the pneumonia could be explained as of hypostatic origin. From the pathologist's point of view, the wide-spread involvement of the lungs argues against such an origin for the lesion. The experiments here reported give no ground for assuming that the heart dilatation precedes the pneumonia; indeed the opposite seems to be the case. The appearance of the pneumonia is more constant than of the heart dilatation, and it would appear accordingly that the heart dilatation depends upon the same circulatory difficulties which determine the same complication in human pneumonias, perhaps in these animals also influenced by the vasoconstrictor effect of the oxygen inhalation.

The general passive congestion noted in practically all the abdominal viscera is such as is seen in cases of heart failure and is to be attributed in these animals to the failure of the heart as a whole or

of its right side. Secondary changes such as cloudy swelling and even fatty degeneration can easily be accounted for by the passive congestion. The appearance of pigment in spleen and lymph nodes is so common in the controls and so inconstant in the oxygen animals that it is not to be regarded definitely as a part of the phenomena of the oxygen poisoning.

The appearance of phagocytes of erythrocytes in the lymph nodes and spleen is more frequent in the oxygen animals than in the controls. This might depend upon some disturbance of the hematopoietic balance such as is supposed to take place in splenectomized animals (25), or it may be the result of the passive congestion in these organs. The studies of the resistance of the erythrocytes to hypotonic salt solutions, the studies of the other features of the blood and of the bone marrow fail to show any distinct general disturbance of the hematopoietic system. The livers of these animals failed to show phagocytosis in the proportion of cases which would be expected were the hematopoietic system disturbed as in blood destruction in splenectomized animals. These facts and the fact that controls showing passive congestion also showed similar phagocytosis of erythrocytes in the lymph nodes and spleen leads to the conclusion that in this series of experiments this phenomenon is due to local blood destruction as the result of passive congestion and perhaps also of the small hemorrhages which frequently are seen in cases of passive congestion. It seems improbable, however, that the pigmentation of these organs, which frequently is so extensive, can be accounted for in the same way because of the short duration of the experiments.

CONCLUSIONS.

In spite of numerous abnormalities or non-experimental lesions in the rabbit certain facts can be considered as established. It has been known for many years that pneumonia is produced by the more or less prolonged inhalation of high partial pressures of oxygen. The studies herein reported show that atmospheres containing 80 to 96 per cent oxygen under normal barometric pressure produce in 24 hours, or more commonly 48 hours, congestion, edema, epithelial degeneration and desquamation, fibrin formation, and finally a pneu-

monia, probably of irritative origin and to be described as a fibrinous bronchopneumonia. The important new points are the time relations of these changes and definition of the type of the pneumonia.

Other studies have noted slight passive congestion, but it is now established that this is to be accounted for in most cases by dilatation of the right side or of both sides of the heart. This congestion affects all the abdominal viscera and is accompanied by certain secondary changes such as cloudy swelling of the parenchymatous organs and phagocytosis of erythrocytes by endothelial cells of the mesenteric lymph nodes.

Although deficiency of oxygen may affect the hematopoietic system, the animals subjected to high oxygen percentages failed to show any demonstrable pathologic changes in blood, spleen, lymph nodes, or bone marrow, except for the presence of congestion.

This study is the first of a comprehensive series projected in and under the direction of the Carnegie Nutrition Laboratory. Different animals and various methods of attack will be employed in the investigation.

BIBLIOGRAPHY.

1. Schmiedehausen, P. G., Die pathologisch-anatomischen Veränderungen der Lungen bei verändertem Sauerstoffgehalt der Atemluft, Inaugural Dissertation, Halle, 1909.
2. Bert, P., La pression barométrique, Paris, 1878.
3. Smith, J. L., The Pathological Effects Due to Increase of Oxygen Tension in the Air Breathed, *Jour. Physiol.*, 1899, xxiv, 19.
4. Heller, R., Mager, W., and von Schrötter, H., Luftdruck-Erkrankungen mit besonderer Berücksichtigung der sogenannten Caissonkrankheit, Vienna, 1900.
5. Hill, L., and Macleod, J. J. R., The Influence of Compressed Air on the Respiratory Exchange, *Jour. Physiol.*, 1903, xxix, 492.
6. Schmiedehausen, P. G., Die pathologisch-anatomischen Veränderungen der Lungen bei verändertem Sauerstoffgehalt der Atemluft, Inaugural Dissertation, Halle, 1909.
7. Schmidt, A., and David, O., Zur Chloroformsauerstoffnarkose, *München. med. Wchnschr.*, 1911, lviii, 25.
8. David, O., Versuche zur Erzeugung von Lungenhyperämie, *Ztschr. f. klin. Med.*, 1912, lxxiv, 404.
9. Bornstein, A., and Stroink, Ueber Sauerstoffvergiftung, *Deutsch. med. Wchnschr.*, 1912, xxxviii, 1495.

10. Schmidt, A., and David, O., Zur Frage der Sauerstoffvergiftung, *Deutsch. med. Wchnschr.*, 1912, xxxviii, 1697.
11. Benedict, F. G., A Respiration Apparatus for Small Animals, *Jour. Biol. Chem.*, 1915, xx, 301.
12. Brünig, A., Ueber Sauerstoffvergiftung, *Deutsch. med. Wchnschr.*, 1912, xxxviii, 1651.
13. Bornstein, A., and Stroink, Ueber Sauerstoffvergiftung, *Deutsch. med. Wchnschr.*, 1912, xxxviii, 1495.
14. Christian, H. A., Smith, R. M., and Walker, I. C., Experimental Cardiorenal Disease, *Arch. Int. Med.*, 1911, viii, 468.
15. LeCount, E. R., and Jackson, L., The Renal Changes in Rabbits Inoculated with Streptococci, *Jour. Infect. Dis.*, 1914, xv, 389.
16. Bunting, C. H., Experimental Anaemias in the Rabbit, *Jour. Exper. Med.*, 1906, viii, 625.
17. Wright, J. H., The Origin and Nature of the Blood Plates, *Boston Med. and Surg. Jour.*, 1906, cliv, 643.
18. Karsner, H. T., and Pearce, R. M., The Relation of the Spleen to Blood Destruction and Regeneration and to Hemolytic Jaundice. IV. A Study, by the Methods of Immunology, of the Increased Resistance of the Red Blood Corpuscles after Splenectomy, *Jour. Exper. Med.*, 1912, xvi, 769.
19. Hill, L., and Macleod, J. J. R., The Influence of Compressed Air on the Respiratory Exchange, *Jour. Physiol.*, 1903, xxix, 492.
20. Smith, J. L., The Pathological Effects Due to Increase of Oxygen Tension in the Air Breathed, *Jour. Physiol.*, 1899, xxiv, 19.
21. Bornstein, A., and Stroink, Ueber Sauerstoffvergiftung, *Deutsch. med. Wchnschr.*, 1912, xxxviii, 1495.
22. Benedict, F. G., and Higgins, H. L., Effects on Men at Rest of Breathing Oxygen-Rich Mixtures, *Am. Jour. Physiol.*, 1911, xxviii, 1.
23. Parkinson, J., The Effect of Inhalation of Oxygen on the Rate of Pulse in Health, *Jour. Physiol.*, 1912, xliv, 54.
24. Retzlaff, K., Der Einfluss des Sauerstoffs auf die Blutcirculation in der Lunge, *Ztschr. f. exper. Path. u. Therap.*, 1913, xiv, 391.
25. Pearce, R. M., and Austin, J. H., The Relation of the Spleen to Blood Destruction and Regeneration and to Hemolytic Jaundice. V. Changes in the Endothelial Cells of the Lymph Nodes and Liver in Splenectomized Animals Receiving Hemolytic Serum, *Jour. Exper. Med.*, 1912, xvi, 780. Karsner, H. T., Amiral, H. H., and Bock, A. V., A Study of the Influence of Splenectomy and of Certain Organs and Organ Extracts on the Hemopsonins of the Blood Serum, *Jour. Med. Research*, 1914, xxx, 383.

EXPLANATION OF PLATES.

PLATE 20.

FIG. 1. Photomicrograph of the lung, showing marked distension of the capillaries with peripheral position of the leukocytes, but without exudation into the alveoli. This condition is not uncommonly seen in control lungs, but it constitutes also the stage of early congestion in the lungs of animals exposed for a short time to atmospheres rich in oxygen.

PLATE 21.

FIG. 2. Photomicrograph of the lung, showing swelling and granular degeneration of attached alveolar epithelium, also moderate desquamation and granular precipitate within the alveoli.

PLATE 22.

FIG. 3. Photomicrograph of the lung, showing advanced desquamation of alveolar epithelium with slight infiltration of lymphocytes and an occasional leukocyte.

PLATE 23.

FIG. 4. Photomicrograph of the lung, showing rich fibrin formation, enclosing in the mesh desquamated epithelium, lymphocytes, and leukocytes.

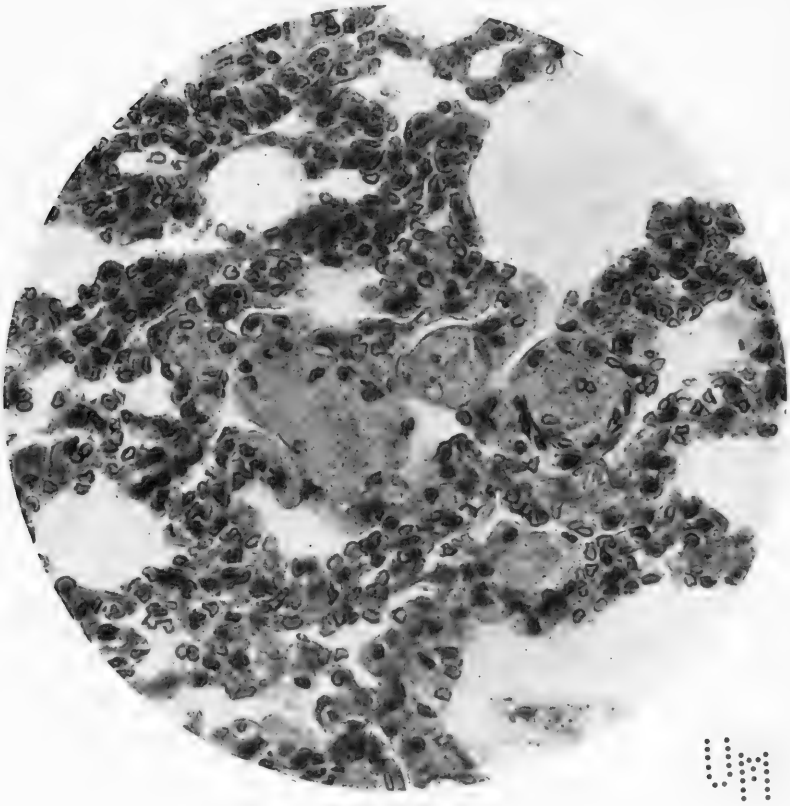

FIG. 1.

(Karsner: Atmospheres Rich in Oxygen.)

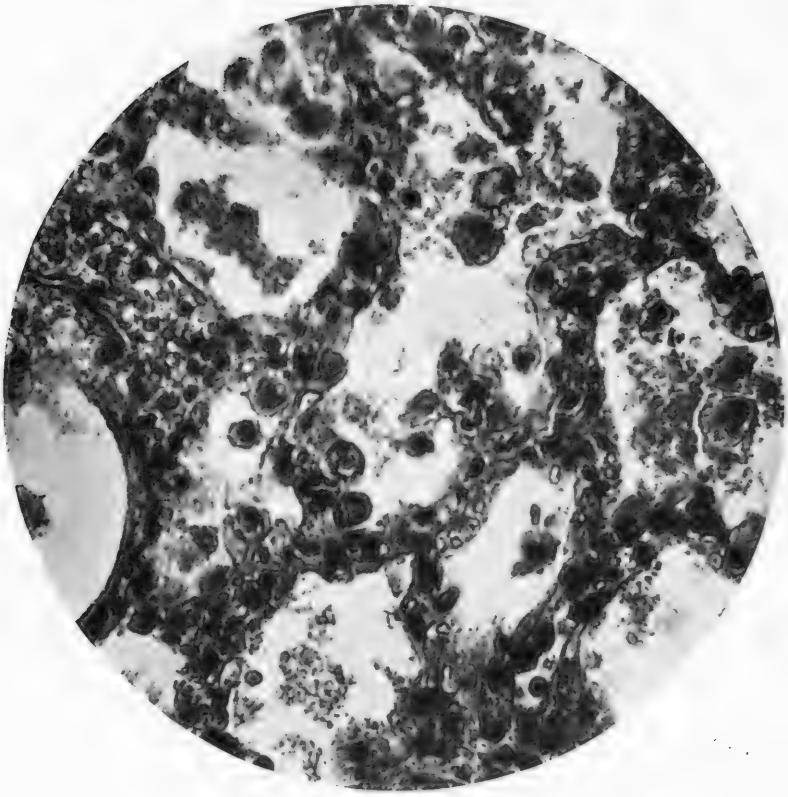

FIG. 2.

(Karsner: Atmospheres Rich in Oxygen.)

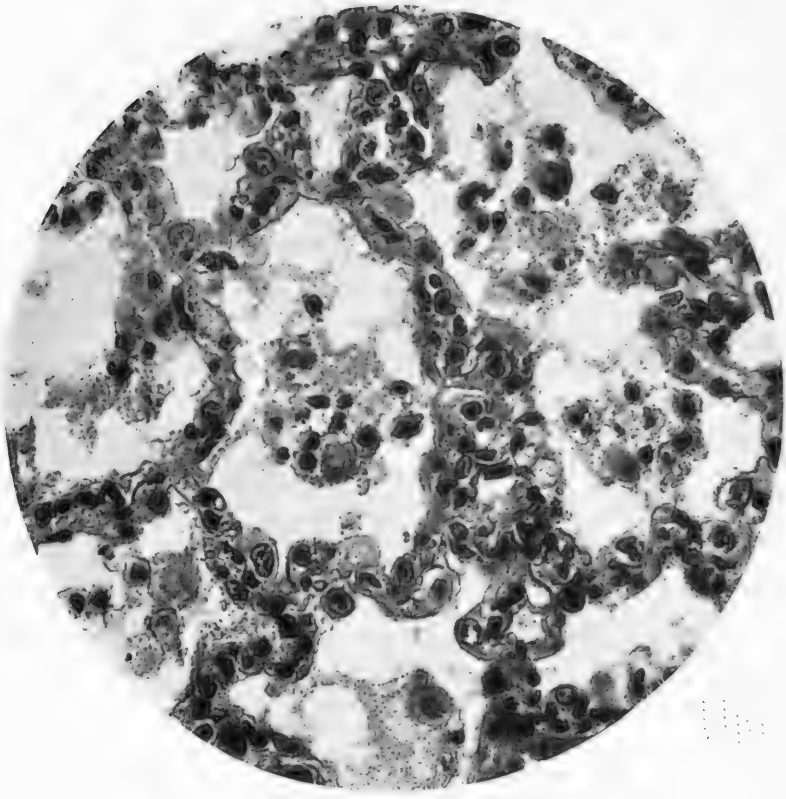

FIG. 3.

(Karsner: Atmospheres Rich in Oxygen.)

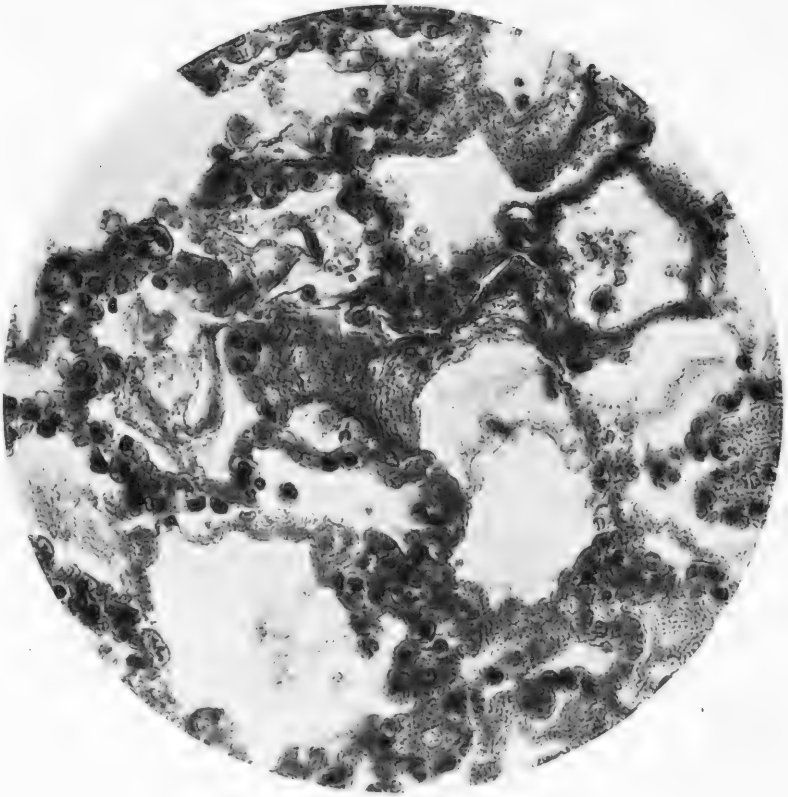

FIG. 4.

(Karsner: Atmospheres Rich in Oxygen.)

THE INHIBITION OF THE TOXICITY OF URANIUM NITRATE BY SODIUM CARBONATE, AND THE PROTECTION OF THE KIDNEY ACUTELY NEPHROPATHIC FROM URANIUM FROM THE TOXIC ACTION OF AN ANESTHETIC BY SODIUM CARBONATE. *

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PLATES 24 AND 25.

(Received for publication, October 25, 1915.)

The first detailed study of the action of the salts of uranium was made by Chittenden and Hutchinson[1] and by Chittenden[2] and Lambert in two publications which appeared in 1887 and 1888. The first of these publications was concerned with the effect of various soluble salts of uranium on the amylolytic and proteolytic ferments occurring in the animal body. The authors showed that in very high dilutions these salts of uranium, and especially the nitrate, exerted an inhibitory effect upon the action of the above mentioned ferments. The second paper, although it was chiefly concerned with the effect of uranium salts upon protein metabolism, gave a brief account of the toxic effect of uranium in so far as the liver and kidney changes were concerned. The ability of uranium to induce a glycosuria was recorded, and the observation was made that the glycosuria usually did not occur until after the appearance of albumin in the urine. The appearance of glucose in the urine was attributed to a loss on the part of the liver of its glycogenolytic function.

Since these publications, uranium has been employed by a large number of investigators in the study of its effect upon metabolism,

* Aided by a grant from the fund for Scientific Research of the American Medical Association.

[1] Chittenden, R. H., and Hutchinson, M. T., The Influence of Uranium Salts on the Amylolytic Action of Saliva and the Proteolytic Action of Pepsin and Trypsin, *Tr. Conn. Acad. Arts and Sc.*, 1885–88, vii, 161.

[2] Chittenden, R. H., and Hutchinson, M. T., Some Experiments on the Physiological Action of Uranium Salts, *Tr. Conn. Acad. Arts and Sc.*, 1888–92, viii, 1.

especially protein metabolism; and on account of its toxic action on the liver and kidney it has been largely used in studying the function of these organs in various stages of poisoning. The toxic effect of uranium for the liver and for the kidney is usually ascribed to the action of the metal as such. The experiments which have been conducted in the present study tend to show that the toxicity of uranium runs parallel with its ability to lead to the formation of various acid bodies, and that if the appearance of these substances in the urine is delayed and their amount diminished there is less evidence of the toxic action of the metal.

The present paper is concerned with the toxic action of uranium nitrate on the kidney, both before and after the use of an anesthetic, and with the inhibition of this toxic action which may be induced by the use of an alkali.

In a recent paper[3] which was concerned with the study of the action of various diuretics in uranium nephritis, it was shown that two factors largely determined the efficiency of these diuretic substances. The first of these factors apparently depended upon the age of the animal, and the second on the form of anesthetic employed in conducting the experiments. Concerning the age of the animal, it was observed that before the use of an anesthetic the older animals gave greater evidence of the toxic effect of uranium than did the younger animals. This increased toxicity of uranium for the older animals was shown by the urine of these animals containing a higher percentage of both albumin and glucose, and by the earlier appearance in the urine, and by the appearance in larger amounts of both acetone and diacetic acid. The animals comprising the above series of experiments were either anesthetized by Gréhant's anesthetic, the active anesthetic ingredients of which are chloroform and alcohol, or by morphine and ether. The animals that received Grehant's anesthetic were in general less responsive to the diuretic substances than were the animals anesthetized by morphine and ether. The adult and old animals of the series which had shown an early appearance of acetone bodies in the urine, upon becoming anesthetized became acutely anuric and remained non-responsive to all diuretic substances.

The histological study of the kidneys of these acutely anuric animals has constantly shown a marked degree of swelling, vacuolation, and necrosis of the epithelium of the convoluted tubules. There was no evidence of any structural

[3] MacNider, W. deB., A Study of the Action of Various Diuretics in Uranium Nephritis, with Special Reference to the Part Played by the Anesthetic in Determining the Efficiency of the Diuretic, *Jour. Pharmacol. and Exper. Therap.*, 1912–13, iv, 491.

change of a degenerative character in the blood vessels of the kidney or in the glomeruli.

Since the publication of this paper other experiments which have been concerned with the difference in the response of animals of different ages to a constant quantity of uranium per kilo, have shown that even though the output of albumin in the urine by these nephropathic animals is of low percentage, if the output of acetone bodies was marked, the animals after being anesthetized either became non-responsive to diuretics or became acutely anuric. The kidneys of these animals have shown changes in the epithelium identical in character to the changes already described.

From the foregoing account it will be seen that there is an apparent connection between the formation of acetone and diacetic acid on the part of an animal and the toxic effect of uranium, and that the animals that show an early appearance of these bodies in the urine are more susceptible to the toxic effect of an anesthetic than the animals that either fail to show the presence of acetone bodies or show these substances in small amounts and late in the uranium intoxication.

It has been shown by Fischer[4] that an accumulation of organic acids, such for instance as lactic acid, is capable of inducing extensive tissue changes; and, also, that the use of an alkali in the form of a carbonate is capable of modifying these changes. With this observation of Fischer's in mind, and with the apparent connection between the toxicity of uranium and the toxicity of an anesthetic, with the appearance in the urine of acetone and diacetic acid, these experiments were planned in order to ascertain if the use of an alkali would in any measure inhibit the toxicity of uranium and the toxic effect of an anesthetic.

Since the commencement of these experiments the work of Graham[5] has appeared, in which he was able to show that all the characteristic features of late chloroform poisoning could be produced by the administration of hydrochloric acid, and that the use of a sodium carbonate solution would greatly inhibit the production of the characteristic lesions which develop in the liver. In his work, Graham has further shown that the ability of various narcotic substances to induce liver necrosis ran parallel with the amount of hydrochloric acid which these substances could theoretically yield in their decomposition outside of the body.

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[4] Fischer, M. H., Oedema, a Study of the Physiology and the Pathology of Water Absorption by the Living Organism, New York, 1910.

[5] Graham, E. A., Late Poisoning with Chloroform and Other Alkyl Halides in Relationship to the Halogen Acids Formed by Their Chemical Dissociation, *Jour. Exper. Med.*, 1915, xxii, 48.

This paper strengthens the views held by Fischer concerning the important part played by various acid bodies in a variety of pathological conditions and of the beneficial effect in some of these conditions which may be obtained by the use of an alkali. It is hoped that the present paper will in some measure serve as additional evidence in this connection.

EXPERIMENTS.

The observations which form the ground for the conclusions obtained in this paper are based upon the study of the toxic effect of uranium nitrate in the dose of 5 mg. per kilo in twenty-four dogs. In previous papers[3,6] it has been shown that the toxicity of uranium is largely dependent upon the age of the animal. As a result of this observation, in conducting these experiments due consideration has been given to this factor. The two animals which have been employed in any one experiment, in which one animal served as a control for the animal receiving the alkaline solution, were either from the same litter, and therefore of the same age, or it was ascertained by careful inquiry that the animals were within a few months of the same age.

The animals were kept in metabolism cages and fed on a diet of bread and a small amount of cooked meat. They were given daily 500 cc. of water by stomach tube. The importance of diet in determining the toxicity of uranium nitrate and other poisons has been shown by Opie.[7] Animals have been shown to be more susceptible to these poisons after a diet rich in meat than following a diet rich in carbohydrate. For this reason the animals which we have employed have received very largely a carbohydrate diet. The withdrawal of the carbohydrates with an increase of the meat of the diet has resulted in an earlier appearance of diacetic acid and acetone in the urine and an increased susceptibility of the animals to anes-

[6] MacNider, W. deB., On the Difference in the Response of Animals of Different Ages to a Constant Quantity of Uranium Nitrate, *Proc. Soc. Exper. Biol. and Med.*, 1913–14, xi, 159.

[7] Opie, E. L., and Alford, L. B., The Influence of Diet upon Necrosis Caused by Hepatic and Renal Poisons. I. Diet and the Hepatic Lesions of Chloroform, Phosphorus, or Alcohol, *Jour. Exper. Med.*, 1915, xxi, 21.

thetics. After a period of observation lasting over two days, during which time the study of the urine had excluded a naturally acquired nephropathy, the animals were given subcutaneously on two successive days 5 mg. of uranium nitrate per kilo.

On each day immediately following the uranium injections, one of the animals that served as a control was given intravenously by the large saphenous vein 25 cc. per kilo of a 0.9 per cent sodium chloride solution, while the other animal was given in the same manner 25 cc. per kilo of a 3 per cent solution of sodium carbonate in 0.9 per cent sodium chloride. The animals were finally given 500 cc. of water by stomach tube and returned to their respective cages. At the end of forty-eight hours the animals were anesthetized by Gréhant's[8] anesthetic in 60 per cent strength. This anesthetic has been chosen in preference to either chloroform or ether as it is given in a definite amount per kilo by stomach and not by inhalation, and for the reason that it induces an anesthesia which is uniform in its depth and duration. This factor is, of course, of much importance in experiments of the character contained in this paper in which the toxicity of the anesthetic has to be considered.

Half an hour after giving the animals morphine, this being the initial step in inducing an anesthesia by Gréhant's method, and just prior to the use of the chloroform-alcohol mixture, the abdominal wall was anesthetized with a 2 per cent solution of cocaine, the bladder exposed, and all the urine expelled. The abdominal incision was then closed. The control animal of the experiment was then given intravenously the sodium chloride solution, while the other animal received an equivalent amount per kilo of a 3 per cent sodium carbonate solution. Immediately following these injections both animals were given, by stomach, Gréhant's anesthetic. The animals were completely anesthetized within twenty to thirty-five minutes. Usually by the end of the second hour the corneal reflex had returned. None of the animals showed any voluntary movements. The anesthesia was allowed to continue in all the animals for two hours and forty-five minutes. At the end of this time the abdominal incision

[8] The animal is given 0.25 cc. per kilo of a 4 per cent solution of morphine. This is followed in half an hour by 10 cc. per kilo of the following mixture: chloroform, 50 cc.; alcohol and water, each 500 cc.

was reopened and the urine that had been formed during the anesthesia was expelled from the bladder and measured. The kidneys and liver were then removed and the animals killed.

The Inhibition of the Toxicity of Uranium Nitrate by Sodium Carbonate.

The inhibition of the toxic effect of uranium by sodium carbonate is shown in the appended tables. A detailed discussion of this effect as evidenced by the various experiments will therefore not be given, but frequent reference to Tables I and II will be made. The tables show, in the first place, that the age of the animal influences the toxic effect of a constant quantity of uranium. If the carbonate animals be excluded from the consideration and only the response of the control animals to the uranium be taken into account, the effect of this factor—the age of the animal—is clearly seen. Thus in Experiment 3, Table I, a control animal four and a half months old, during the first twenty-four hour period following the first uranium injection, the urine contained only 0.5 gm. of albumin per liter, no glucose, and no acetone. At the end of the second twenty-four hour period, following the second injection of uranium, the urine contained only 1 gm. of albumin, 0.85 per cent glucose, and a trace of acetone. However, in Experiment 15, Table II, also a control animal, but an animal between four and five years old, the urine of the first twenty-four hour period contained 1.25 gm. of albumin, 1.67 per cent of glucose, and gave a pronounced test for acetone. At the end of the second twenty-four hour period the albumin had reached 4.75 gm. per liter, the percentage of glucose had increased to 2.06, and the urine contained not only acetone in large amount but also diacetic acid. The inhibition of the toxic effect of uranium by sodium carbonate in animals of the same age is shown in the accompanying tables by comparing the difference in the output of albumin, glucose, and acetone by the control animals with the carbonate animals.

It will be seen by referring to the tables of experiments that the animals that receive the carbonate protection show uniformly in the urine of both the first and second twenty-four hour periods a smaller

output of albumin and a lower percentage of glucose than do the animals of the same age which have not received the carbonate but which have received an equivalent volume per kilo of sodium chloride solution. It will also be seen that in the carbonate animals, with one exception, acetone was never present in the urine of the first twenty-four hour period, whereas in the control animals acetone was present in the urine of the first twenty-four hours in all animals over one year of age. In the above mentioned exception, the amount of acetone in the urine was exceedingly small. It was only detected by finding in the distillate used for the test a few characteristic iodoform crystals.

A study of the experiments shows further that with the appearance of acetone and diacetic acid in the urine, whether the animal be a control animal or an animal protected by the carbonate, there develops with the appearance of these bodies evidence of an increase in the intoxication which is shown by a rise in the percentage of glucose in the urine and in so far as the kidney is concerned in the intoxication, by an increase in the output of albumin.

The control animals have shown uniformly an earlier albuminuria than have the carbonate animals. In the urine of many of the control animals albumin has been present within eight to twelve hours after the first injection of uranium. This is true for all the control animals over one year of age. It will be noted that it is with the animals of this age that the acetone bodies first make their appearance in the urine of the first twenty-four hour period.

In the carbonate animals, however, the appearance of albumin n the urine has been delayed until the end of the first period or into the second period of twenty-four hours. With the carbonate animals it is not until this second twenty-four hour period, with the one exception which has been given, that the acetone bodies make their appearance in the urine.

From the foregoing survey of the experimental data it would appear that the toxicity of uranium is associated with the ability of the metal to lead to the production of acetone bodies, and that when sodium carbonate is employed and the formation of these bodies is delayed and their amount lessened there is less evidence of the toxic effect of the metal. The use of the carbonate in an old animal places

such an animal, in so far as its response to uranium is concerned, and in so far as the toxic effect of an anesthetic is concerned, in the relative position of a young animal in which both uranium and an anesthetic show less evidence of their toxic effect.

The following experiments are selected to illustrate the different points which have been brought out in the general review of the experiments which has just been made. Two sets of experiments will be employed: one set from the younger animals and one set from the older animals of the series.

Experiments 5 and 6, Table I, were carried out in animals between ten and twelve months old. The urine of the control animal, Experiment 5, during the first twenty-four hour period showed only a trace of albumin and glucose and no acetone. During the second period of twenty-four hours the animal developed an acetonuria, albumin was present in the amount of 1.1 gm. per liter, and glucose was present in 1.43 per cent. In Experiment 6, the animal was given the injections of sodium carbonate. The urine of the first twenty-four hour period showed a trace of albumin, no glucose, and no acetone. The urine of the second period showed only a trace of acetone, only 0.7 gm. of albumin per liter, and glucose in a lower percentage than was found in the urine of the control animal. The output of glucose at the end of the second day was 0.142 per cent. Experiments 15 and 16, Table II, were carried out in animals between four and five years of age. The urine of the control animal, Experiment 15, showed within the first twenty-four hour period acetone, albumin 1.25 gm. per liter, and 1.67 per cent of glucose. During the second twenty-four hours the amount of acetone had greatly increased and diacetic acid was present in the urine. The albumin had increased to 4.75 gm. per liter, while glucose was present in 2.06 per cent. The urine of the carbonate animal, Experiment 16, was free from acetone for the first twenty-four hours, there was no glucose in the urine, and only a trace of albumin. During the second twenty-four hour period the urine contained a trace of acetone, the output of albumin was low, 0.85 gm., and the percentage of glucose was only 0.93 per cent.

These two sets of experiments serve to show the difference in the toxicity of uranium in the animals of different ages, the inhibition

TABLE I.

The Inhibition of the Toxicity of Uranium Nitrate by Sodium Carbonate.

No. of experiment.	Age.	Weight.	Water in 24 hrs.	Uranium, 0.9% NaCl or 3% Na_2CO_3, 25 cc. per kilo.	Urine in 1st 24 hrs.	Albumin per liter.	Glucose.	Acetone.	Uranium, 0.9% NaCl or 3% Na_2CO_3, 25 cc. per kilo.	Water in 24 hrs.	Urine in 2d 24 hrs.	Albumin per liter.	Glucose.	Acetone.	Anesthetic, 0.9 % NaCl or 3 % Na_2CO_3, 25 cc. per kilo.	Duration of anesthesia.	Urine during period of anesthesia.
		kg.	*cc.*		*cc.*	*gm.*	*per cent*			*cc.*	*cc.*	*gm.*	*per cent*			*hrs.*	*cc*
1, control.	15 wks.	4.0	250	5 mg. per kg. 0.9% NaCl	212	Trace.	None.	None.	5 mg. per kg. 0.9% NaCl	250	130.5	0.75	0.71	None.	Gréhant's 60%, 0.9% NaCl	2¾	20
2, carbonate.	15 "	4.56	250	5 mg. per kg. 3% Na_2CO_3	320	None.	"	"	5 mg. per kg. 3% Na_2CO_3	250	352	0.25	0 1	"	Gréhant's 60% 3% Na_2CO_3	2¾	29
3, control.	4½ mos.	3.71	400	5 mg. per kg. 0.9% NaCl	385	0.5	"	"	5 mg. per kg. 0.9% NaCl	400	444	1.0	0.85	Acetone present.	Gréhant's 60%, 0.9% NaCl	2¾	2.5
4, carbonate.	4½ "	4.55	400	5 mg. per kg. 3% Na_2CO_3	370	None.	"	"	5 mg. per kg. 3% Na_2CO_3	400	567	0.25	0.19	None.	Gréhant's 60%, 3% Na_2CO_3	2¾	47.5
5, control.	10–11 mos.	7.79	500	5 mg. per kg. 0.9% NaCl	795	Trace.	Trace.	"	5 mg. per kg. 0.9% NaCl	500	1,040	1.1	1.43	Acetone present.	Gréhant's 60%, 0.9% NaCl	2¾	36
6, carbonate.	10–11 "	15.98	500	5 mg. per kg. 3% Na_2CO_3	745	"	None.	"	5 mg. per kg. 3% Na_2CO_3	500	900	0.7	0.142	Trace of acetone.	Gréhant's 60%, 3% Na_2CO_3	2¾	168
7, control.	1 yr.	7.83	500	5 mg. per kg. 0.9% NaCl	1,020	0.25	0.5	Trace.	5 mg. per kg. 0.9% NaCl	500	585	2.1	1.93	Present, heavy. Diacetic acid.	Gréhant's 60%, 0.9% NaCl	2¾	0
8, carbonate.	1 "	12.91	500	5 mg. per kg. 3% Na_2CO_3	95	None.	None.	None.	5 mg. per kg. 3% Na_2CO_3	500	682	0.4	0.63	Trace of acetone.	Gréhant's 60%, 3% Na_2CO_3	2¾	101

TABLE II.

The Inhibition of the Toxicity of Uranium Nitrate by Sodium Carbonate.

No. of experiment.	Age.	Weight.	Water in 24 hrs.	Uranium, 0.9% NaCl or 3% Na_2CO_3, 25 cc. per kilo.	Urine in 1st 24 hrs.	Albumin per liter.	Glucose.	Acetone.	Uranium, 0.9% NaCl or 3% Na_2CO_3, 25 cc. per kilo.	Water in 24 hrs.	Urine in 2d 24 hrs.	Albumin per liter.	Glucose.	Acetone and diacetic acid.	Anesthetic, 0.9% NaCl or 3% Na_2CO_3, 25 cc. per kilo.	Duration of anesthesia.	Urine during period of anesthesia.
	yrs.	*kg.*	*cc.*		*cc.*	*gm.*	*per cent*			*cc.*	*cc.*	*gm.*	*per cent*			*hrs.*	*cc.*
9, control.	1½–2	8.83	500	5 mg. per kg. 0.9% NaCl	820	1.9	1.46	Present.	5 mg. per kg. 0.9% NaCl	500	492	2.1	1.48	Present, heavy. Diacetic acid.	Gréhant's 60%, 0.9% NaCl	2¾	0.6
10, carbonate.	1½–2	11.61	500	5 mg. per kg. 3% Na_2CO_3	600	0.4	0.12	Trace.	5 mg. per kg. 3% Na_2CO_3	500	485	0.6	0.5	Trace of acetone.	Gréhant's 60%, 3% Na_2CO_3	2¾	114
11, control.	1½–2	9.61	500	5 mg. per kg. 0.9% NaCl	475	1.0	0.52	Present.	5 mg. per kg. 0.9% NaC_3	500	195	1.75	1.6	Present, heavy. Diacetic acid.	Gréhant's 60%, 0.9% NaCl	2¾	9
12, carbonate.	1½–2	15	500	5 mg. per kg. 3% Na_2CO_3	754	0.5	0.43	None.	5 mg. per kg. 3% Na_2CO_3	500	480	0.65	0.66	Trace of acetone.	Gréhant's 60%, 3% Na_2CO_3	2¾	95
13, control.	4–5	16.17	500	5 mg. per kg. 0.9% NaCl	1,140	1.15	0.5	Present.	5 mg. per kg. 0.9% NaCl	500	613	3.85	1.95	Present, heavy. Diacetic acid.	Gréhant's 60%, 0.9% NaCl	2¾	13
14, carbonate.	4–5	16.83	500	5 mg. per kg. 3% Na_2CO_3	485	0.3	None.	None.	5 mg. per kg. 3% Na_2CO_3	500	618	0.4	0.37	Trace of acetone.	Gréhant's 60%, 3% Na_2CO_3	2¾	151
15, control.	4–5	11.26	500	5 mg. per kg. 0.9% NaCl	1,137	1.25	1.67	Present.	5 mg. per kg. 0.9% NaCl	500	763	4.75	2.06	Present, heavy. Diacetic acid.	Gréhant's 60%, 0.9% NaCl	2¾	10
16, carbonate.	4–5	15.15	500	5 mg. per kg. 3% Na_2CO_3	1,225	0.25	None.	None.	5 mg. per kg. 3% Na_2CO_3	500	1,079	0.85	0.93	Trace of acetone.	Gréhant's 60%, 3% Na_2CO_3	2¾	95
X, uranium dissolved in 3% Na_2CO_3.	2	13.55	500	5 mg. per kg. in 3% Na_2CO_3	775	1.3	0.94	Present.	5 mg. per kg. in 3% Na_2CO_3	500	758	3.4	2.03	Present, heavy. Diacetic acid.	Gréhant's 60%	2¾	3.5

of this toxic effect by the use of the carbonate, and the relation which exists between the appearance in the urine of acetone bodies and an increase in the toxic action of the metal.

The Protection of the Kidney Acutely Nephropathic from Uranium from the Toxic Action of an Anesthetic by Sodium Carbonate.

The effect of this anesthetic on the pathology of the kidney in acute uranium nephritis, and through this effect its ability to render the kidney non-responsive to various diuretics has been shown by Pearce, Hill, and Eisenbrey,[9] and by several publications which have originated from this laboratory.[10,11]

In these later experiments it has been shown that when dogs of different ages were rendered nephropathic by uranium nitrate in the dose of 6.7 mg. per kilo and anesthetized by Gréhant's anesthetic in 60 per cent strength, the younger animals remained diuretic and responsive to such substances as caffeine, theobromine, and various salts in different molecular concentrations, while the older animals became anuric as soon as they were anesthetized and remained anuric and non-responsive to these diuretic substances. It was further shown that the failure of these substances to be of diuretic value was not dependent upon any lack of response on the part of the vascular mechanism of the kidney and that it was not due to any histological changes in this mechanism, but that the failure of these bodies to induce diuresis was constantly associated with the development in the kidney of various degrees of degeneration of the epithelium, and especially of the epithelium of the convoluted tubules.

With these observations in mind of the definite toxic effect that Gréhant's anesthetic has for the kidney acutely nephropathic from

[9] Pearce, R. M., Hill, M. C., and Eisenbrey, A. B., Experimental Acute Nephritis: the Vascular Reactions and the Elimination of Nitrogen, *Jour. Exper. Med.*, 1910, xii, 196.

[10] MacNider, W. deB., The Effect of Different Anesthetics on the Pathology of the Kidney in Acute Uranium Nephritis, *Jour. Med. Research*, 1913, xxviii, 403.

[11] MacNider, W. deB., The Vascular Response of the Kidney in Acute Uranium Nephritis,—the Influence of the Vascular Response on Diuresis, *Jour. Pharmacol. and Exper. Therap.*, 1914–15, vi, 123.

uranium, and with the observation that has been made in this paper of the ability of a carbonate to inhibit the toxic action of uranium for the kidney prior to an anesthetic, both the control and carbonate animals were anesthetized by Gréhant's mixture, and the effect of the anesthetic on the kidney was determined; first, by the comparative ability of the kidneys of the control and carbonate animals to form urine during an anesthesia of two hours and forty-five minutes, and, secondly, by the difference in the severity of the pathological changes induced in the kidneys of the respective animals by the anesthetic.

A study of the tables of experiments shows quite clearly that in so far as the total output of urine is concerned during the period of anesthesia, the formation of urine is greatly favored in the animals that have been given prior to the anesthetic a solution of sodium carbonate, while the control animals that have received an equivalent volume per kilo of sodium chloride solution either secrete a much smaller amount of urine during the same period, or they become completely anuric.

For example, in Experiments 5 and 6, Table I, the animals being between ten and twelve months old, the control animal during the period of anesthesia formed 36 cc. of urine, while the carbonate animal during the same period formed 168 cc. This difference in the output of urine in the control, as compared with the carbonate animals, is seen to a more marked degree in the older animals of the experiments and serves to confirm the observation previously made of the increased toxicity of both uranium and of an anesthetic for these animals. In Experiments 9 and 10, Table II, in animals between one and a half and two years of age, the control animal during the period of anesthesia formed only 0.6 of 1 cc. of urine, while the carbonate animal had an output of urine of 114 cc.

The pathology of the kidneys of the control as compared with the carbonate animals shows the following differences. The kidneys of the control animals have shown a pronounced fatty degeneration of the loops of Henle. The kidneys of the carbonate animals have shown in all experiments less fatty degeneration of this epithelium. The most marked difference in the pathological response of the kidneys of the control animals, as compared with the changes in the kidneys

of the carbonate animals, is seen in the difference in the degree of involvement of the epithelium of the convoluted tubules. In the control animals the epithelium of these tubules shows severe injury (Fig. 1). The cells are greatly swollen and the nuclei are faint and frequently fragmented. In other of the tubules both the nucleus and cytoplasm of the cell have become necrotic. The epithelium of the collecting and junctional tubules is strikingly spared.

In the carbonate animals the epithelium of the convoluted tubules shows but slight injury and these changes are early. The terminal effect of the anesthetic on these cells, such, for instance, as a fairly complete necrosis, has not occurred in the twelve animals of the series which have received the carbonate protection.

In general the cytoplasm and nucleus of the cells of these tubules stain well. The cytoplasm is not infrequently granular, and in a few of the tubules the cells have undergone some swelling (Fig. 2). The vascular pathology of the kidney in both the control and carbonate animals has consisted in a fairly severe engorgement of the glomerular vessels, but without the appearance of any exudate into the glomerulus and without any structural changes in the endothelium of the capillaries.

From the foregoing account the conclusion appears allowable that the kidney of an animal acutely nephropathic from uranium is protected against the toxic effect of an anesthetic by the use of sodium carbonate, and, furthermore, that this protection is dependent upon the histological preservation of the renal epithelium and especially of the epithelium of the convoluted tubules.

DISCUSSION.

No attempt will be made to explain the exact mechanism through which uranium exerts its toxicity, or to explain the differences between young tissue and old tissue which apparently give to the younger animals a relative immunity to an amount of uranium which in an older animal serves as a severe toxic agent. Even though the exact mechanism of the toxic action of uranium may not be clear, we know enough of the results of its toxic effect to warrant a brief discussion of the origin of some of the tissue changes and changes in the urine.

The earliest indication of the toxicity of uranium is shown by albumin in the urine. There also appears in the urine either prior to the albumin or within a few hours of the first trace of albumin, acetone and, later, diacetic acid. Generally within twelve hours after the development of an albuminuria the animal develops a glycosuria. So far as we have been able to learn there is no attempt to explain the nephritis from uranium except through the effect of the metal on the kidney.

The glycosuria has either been explained by assuming that the glycogenolytic function of the liver is interfered with, allowing an increase in blood sugar, which finds its escape in the urine, or that the glycosuria is purely of renal origin. That it is dependent upon an injury to the kidney, and that without any increase in the amount of blood sugar the animal becomes glycosuric, is the explanation given by Wallace[12] in his study of uranium glycosuria. Neither of these explanations serves to explain the severe general toxic effect of uranium which is seen in the rapid loss of weight by the animals, or the difference in the toxicity of uranium for animals of different age, and the early appearance in the urine of acetone and diacetic acid. It appears more likely that in its toxic action, uranium has a more general effect upon cell metabolism, resembling in some respects the action of hydrocyanic acid, and that the albuminuria, glycosuria, the formation of acetone bodies, and the marked loss in weight by the intoxicated animals is an expression of this interference in a wide variety of cells.

From experiments that are now being conducted in this laboratory and from the previous work of others, we are more inclined to this explanation of the toxic effect of uranium, than to ascribe its toxicity to the direct effect of the metal on any one or more groups of cells, as, for instance, the liver or the kidney.

It has been shown by Chittenden and Hutchinson[1] in their experiments on the action of various uranium salts on certain ferments, that the nitrate exerted in very high dilutions a marked inhibitory effect upon the action of both ptyalin and pepsin.

[12] Wallace, G. B., and Myers, H. B., Uranium Glycosuria, *Jour. Pharmacol. and Exper. Therap.*, 1913–14, v, 511.

It is possible that uranium nitrate, as used in the experiments recorded in this paper, may exert a similar inhibitory effect on the oxidative enzymes of a variety of cells, and that through this action uranium induces histological changes which are common to different cells, such as acute swelling, and causes the appearance in the urine of various abnormal bodies. It would then follow from the facts observed in the experiments, that the oxidative capacity of the young animals is greater than the oxidative capacity of the older animals; for when both types of animals have been given the same amount of uranium per kilo, there is less evidence of its inhibitory effect on processes of oxidation in the young animals than in the older animals. The younger animals show a lower percentage of glucose in the urine, a later appearance of acetone bodies in the urine, and, as a result of the milder grade of nephritis, there is a lower output of albumin.

Experiments that are now being conducted have as their object a study of the oxidative capacity of the blood and various tissue juices in normal animals of different ages and in animals of different ages nephropathic from a constant quantity of uranium per kilo. They tend to substantiate the purely hypothetical explanation which has been offered for the observation that animals of different ages vary in their susceptibility to uranium and that this toxic effect of uranium is constantly associated with the appearance in the urine of acetone and diacetic acid.

In this paper it has not been shown that these bodies acting as such are the cause of the kidney damage. Other bodies similar in character may be formed in the kidney cells which are in large measure responsible for their degeneration. It has, however, been shown that the time of the appearance of these substances and the relative amount in which they appear is an index of the severity of the uranium intoxication, and that if their appearance is delayed by the use of sodium carbonate there is less evidence of the toxic effect of uranium on the kidney both prior to an anesthetic and also after a period of anesthesia.

Before arriving at this conclusion concerning the action of the carbonate it was necessary to exclude the possible detoxicating effect which sodium carbonate might have through its action on uranium nitrate, by the formation of a less toxic salt. The fact that the

different salts of uranium vary in their ability to inhibit ferment action has been shown by Chittenden and Hutchinson.[1] They were able to show that uranium acetate caused more inhibition of the action of ptyalin than did the nitrate, and they ascribed this difference to the greater acidity of the former salt. Likewise, the oxychloride of uranium showed more inhibition of the action of ptyalin than did uranium sulphate.

In order to exclude the possibility of the toxic nitrate of uranium having been detoxicated by the use of sodium carbonate, two animals were given injections of 5 mg. per kilo of uranium nitrate in which the solvent for the uranium was a 3 per cent solution of sodium carbonate in 0.9 per cent sodium chloride. As will be seen by referring to the last experiment of Table II (Experiment X), the toxic effect of uranium is in no way diminished when employed in a 3 per cent solution of the carbonate.

SUMMARY.

1. The toxicity of uranium in animals of different ages is associated with the power of the metal to lead to the formation of organic acids, as, for instance, diacetic acid and also acetone.

2. The power of sodium carbonate to lessen the toxicity of uranium depends upon its power to delay the formation of such bodies and to cause their appearance in the urine in lessened amounts, and does not depend upon the power of the carbonate to detoxicate the metal.

3. The protection of the kidney by the carbonate, which is shown by the kidney being functionally much more active during an anesthesia than the kidney of a control animal, and by the lack of fatty degeneration, acute swelling, and necrosis of the renal epithelium which is constantly seen in the unprotected kidneys, is probably dependent upon two factors: the neutralization of organic acids formed prior to and during the anesthesia, and the neutralization of hydrochloric acid which Graham[5] has shown to be liberated by chloroform during an anesthesia induced by this substance.

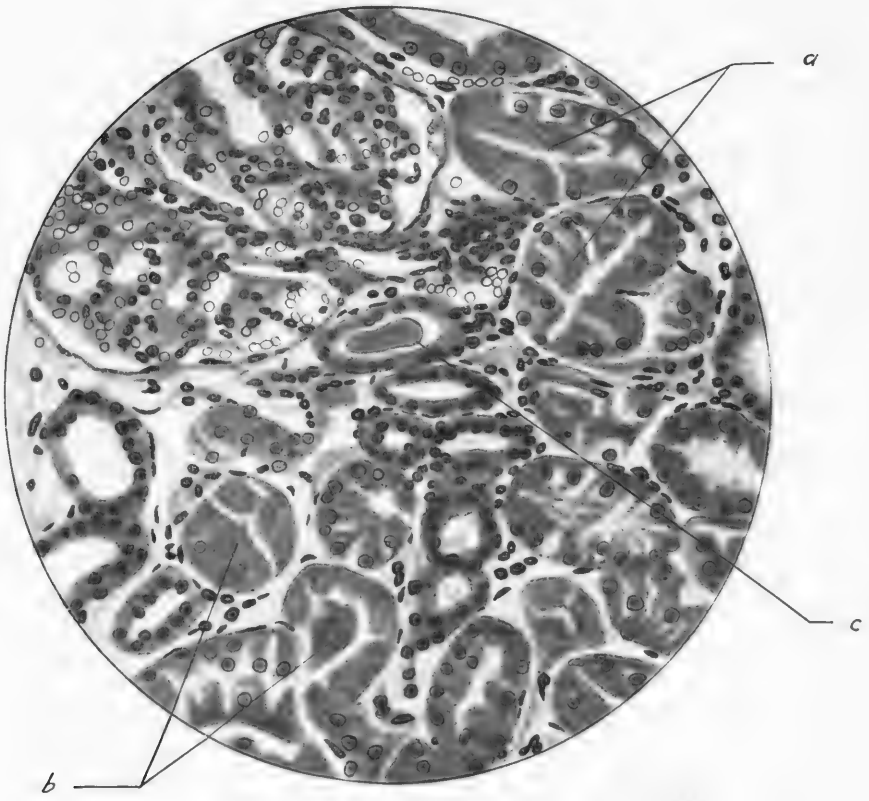


FIG. 1.

(MacNider: Inhibition of Toxicity of Uranium.)

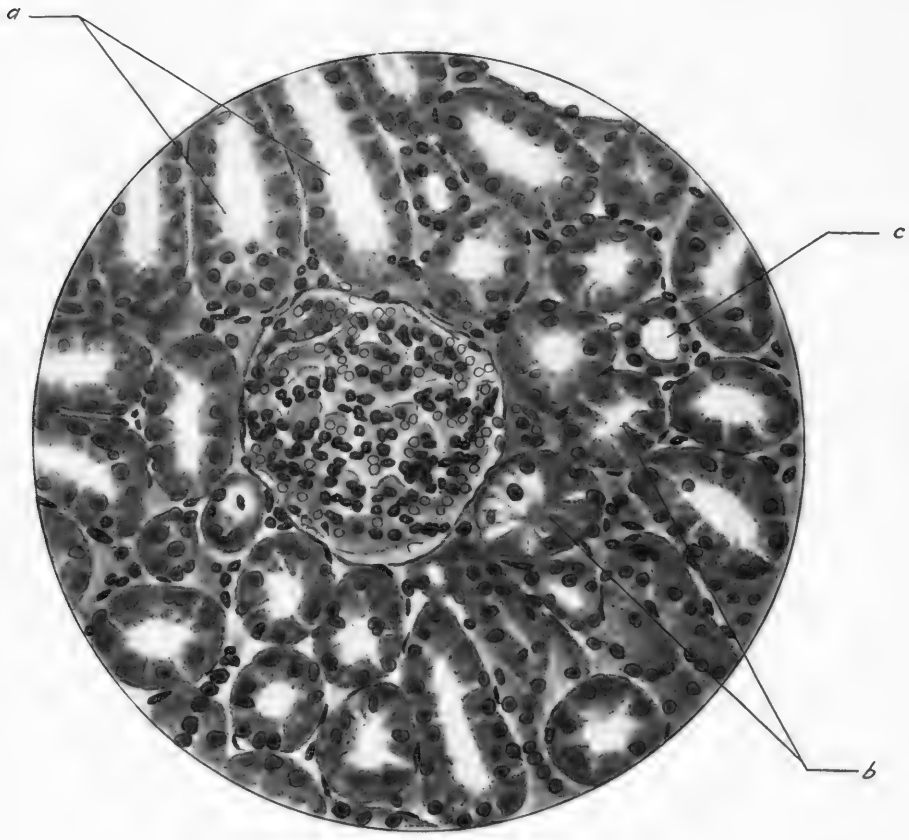


FIG. 2.

(MacNider: Inhibition of Toxicity of Uranium.)

EXPLANATION OF PLATES.

PLATE 24.

FIG. 1. Camera lucida drawing, Leitz oc. 1, obj. 6. The figure is from the kidney of the control animal, Experiment 9. The glomerular capillaries are fairly well filled with blood. In general the epithelium of the convoluted tubules shows either a severe grade of swelling with a commencing necrosis, *a*, or the epithelium is completely necrotic, *b*. The junctional tubules are not involved. One of these tubules, shown at *c*, contains a cast. The kidney formed only 0.6 of 1 cc. of urine during the period of anesthesia.

PLATE 25.

FIG. 2. Camera lucida drawing, Leitz oc. 1, obj. 6. The figure is from the kidney of the carbonate animal of Experiment 10. The glomerulus shows a fairly severe engorgement. The capillaries fill the space enclosed by the capsule. The epithelium of the convoluted tubules in general stains uniformly and none of the cells are necrotic. There is shown at *a* a shrinkage of the epithelium which is frequently seen in hyperactive kidneys. At *b* an early swelling of the epithelium is shown but without necrosis. At *c* is shown in cross-section a junctional tubule, the epithelium of which is normal. The kidney formed 114 cc. of urine during the period of anesthesia.

INTRATESTICULAR IMPLANTATION OF THE FLEXNER-JOBLING RAT CARCINOMA.

By WILLIAM H. WOGLOM, M.D.

(*From Columbia University, George Crocker Special Research Fund, New York.*)

PLATE 26.

(Received for publication, November 4, 1915.)

It is generally agreed that the propagable tumors of mice and rats will proliferate in practically all the organs[1] of the body, including the testis, though the transplantability of the Flexner-Jobling rat carcinoma into this gland has not yet been definitely proved. Thus, Flexner and Jobling[2] said that their attempts to transplant this tumor into the testis had met with but indifferent success, while Levin[3]

[1] The bibliography up to 1913 is to be found in Woglom, W. H., Studies in Cancer and Allied Subjects. The Study of Experimental Cancer, New York, 1913, i. Other references are: Kraus, R., Ranzi, E., and Ehrlich, H., *Wien. klin. Wchnschr.*, 1909, xxii, 1653 (liver, omentum, and testis). Graf, R., *Centralbl. f. allg. Path. u. path. Anat.*, 1910, xxi, 723 (various organs, including the bones). Stumpf, R., *Beitr. z. path. Anat. u. z. allg. Path.*, 1910, xlvii, 571 (kidney). Levin, I., *Jour. Exper. Med.*, 1911, xiii, 604; 1912, xvi, 155 (various organs, including the brain and the bone marrow). Brancati, R., *Tumori*, 1911, i, 189 (various organs). Ruben, L., *Arch. f. Ophth.*, 1912, lxxxi, 199. Da Fano, C., *Folia neuro-biol.*, 1912, vi, 109 (brain). Uhlenhuth, P., *Deutsch. med. Wchnschr.*, 1913, xxxix, 1859 (testis and brain). Keysser, F., *Wien. klin. Wchnschr.*, 1913, xxvi, 1664; *Ztschr. f. Chemotherap., Ite Teil, Orig.*, 1914, ii, 188 (various organs, including the eye). Citron, H., *Ztschr. f. Immunitätsforsch., Orig.*, 1912, xv, 1; *Centralbl. f. Bakteriol., Ite Abt., Orig.*, 1914, lxxii, 328 (wall of stomach). Happe, *Ber. ü. d. xxxix. Versamml. d. ophthalmol. Gesellsch.*, Heidelberg, 1913, 407 (eye). Grignolo, F., *Gior. d. r. Accad. di med. di Torino*, 1914, lxxvii, 285 (eye). Ebeling, E., *Ztschr. f. Krebsforsch.*, 1914, xiv 151 (brain). A detailed account of Keysser's work is given by Hegner, C. A., an ophthalmologist in *München. med. Wchnschr.*, 1913, lx, 2722.

[2] Flexner, S., and Jobling, J. W., *Monographs of The Rockefeller Institute for Medical Research*, 1900, No. 1, 35.

[3] Levin, I., *Jour. Exper. Med.*, 1912, xvi, 149, 155; 1912, xv, 163.

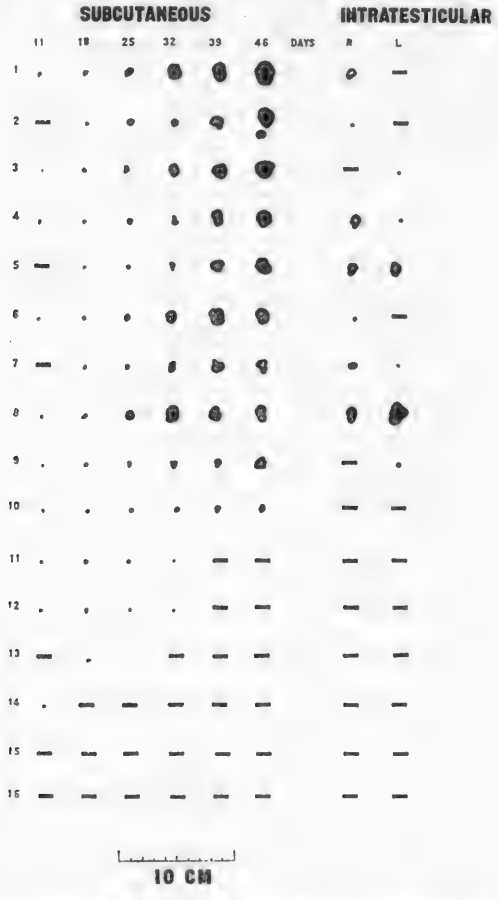
wrote that he had failed entirely to inoculate it into this organ. The failure can not be ascribed to any technical factor, because the Jensen rat sarcoma, transplanted by the same method, grew vigorously in the testis.

Since so many authors have reported the successful inoculation of other tumors at this site, it would appear from the experiments just cited that the Flexner-Jobling carcinoma is an exception to a general rule. Actually, however, it is not, since the experiments about to be described prove that this tumor grows well in the testis. A series of preliminary transplantations, which it is not necessary to reproduce here, demonstrated that the inoculation percentage of the Flexner-Jobling tumor in this gland is almost equal to the subcutaneous inoculation percentage, and that subcutaneous and intratesticular grafts in the same rat generally tend to fail or succeed together.

Although grafts do succeed, therefore, in proliferating in the testis, the resulting tumors do not attain quite the dimensions of those growing in the subcutaneous tissues. Why this should be so, it is impossible to explain. It is not true of all the parenchymatous organs, at any rate, for tumors inoculated into the kidney, for instance, attain dimensions equal to those of axillary neoplasms, as has been pointed out by Woglom.[4] The greater size of the subcutaneous growths is not apparent merely (explicable, that is, by the inclusion of skin at the weekly measurements), for in several experiments where the subcutaneous tumors were measured at autopsy, the skin was found to make but a negligible difference. The testis can not be regarded, in itself, as an unfavorable soil, for the ingrowth of blood vessels and fibroblasts during the first few days following implantation is not less marked than in the subcutaneous tissues (Fig. 1); yet it is possible, of course, that the blood supply in this gland finally becomes inadequate to satisfy the incessant requirements of a rapidly growing tumor. The presence of a growth in the axilla will not account for the smaller size of intratesticular neoplasms, since both tumors were inoculated at the same time; and ample evidence has been accumulated to show that two grafts, simultaneously implanted, do not affect one another. The question of age does not arise, for

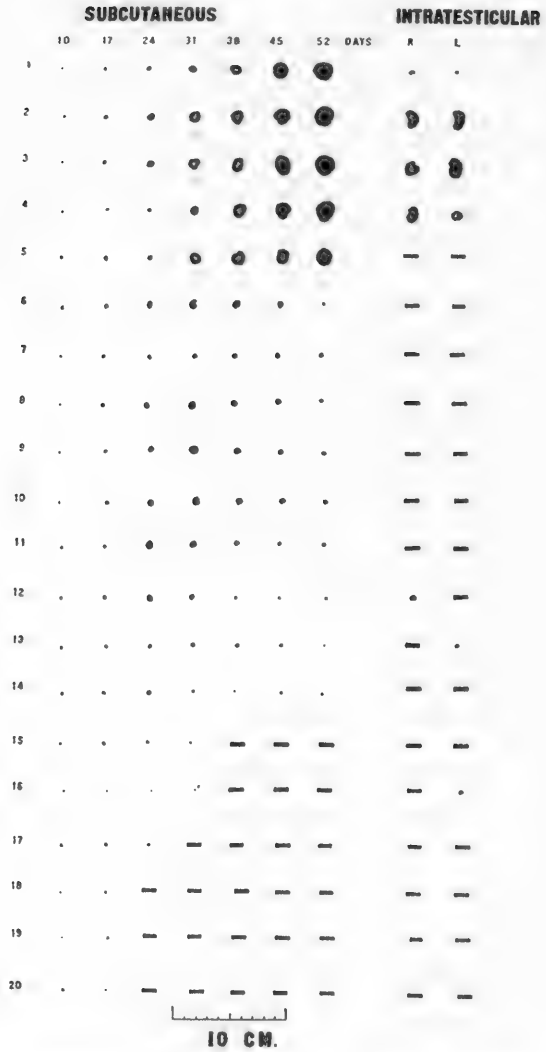
[4] Woglom, W. H., *Lancet*, 1911, ii, 92.

the animals were all young adults. The pressure of the tunica albuginea is the only remaining possibility among those that come readily



TEXT-FIG. 1. $\frac{FRC}{13E}$. Average weight of rats, 99 gm.

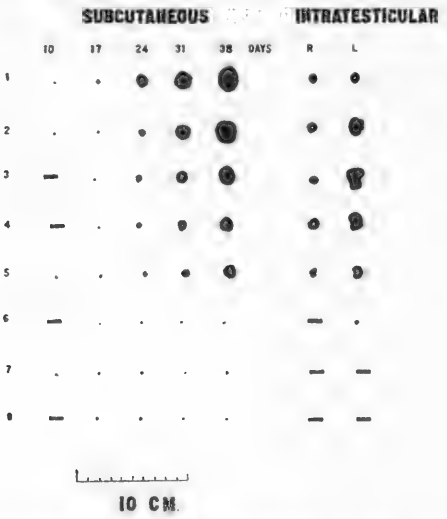
to mind; but the following experiments (Text- figs. 1 to 6) prove that this factor does not explain entirely the smaller size of intratesticular growths.



TEXT-FIG. 2. $\frac{FRC^1}{14GG}$. Average weight of rats, 79 gm.

TEXT-FIG. 3. $\frac{FRC}{14Z}$. Average weight of rats, 76 gm.

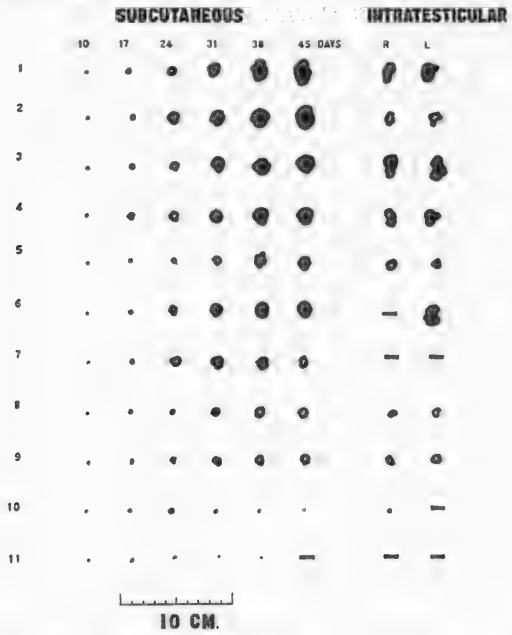
Six groups of young adult rats averaging 77 gm. in weight (except in experiments $\frac{\text{FRC}}{13\ \text{E}}$ where the weight was 99 gm., and $\frac{\text{FRC}}{13\ \text{O}}$ where the rats were not weighed), were inoculated in both testes and in the right axilla, each series at the same sitting and with an equal dose (0.01 gm. by the needle method) of the same tumor. From three to seven days afterward, the tension in the left testis was lowered by incising the tunic through an abdominal incision under ether anes-



TEXT-FIG. 4. $\frac{\text{FRC}}{13\text{C}}$. Average weight of rats, 77 gm.

thesia, while the right testis was left undisturbed as a control. The figures show the results when the rats were autopsied, from thirty-eight to fifty-two days after inoculation, the two columns to the right representing the growths in the right and left testes. The tumors in the opened (left) gland were not invariably larger than those in the unopened, as they would be were tension alone responsible for the smaller size of intratesticular growths. That the release of pressure may occasionally exert some slight favorable influence, however, is suggested by the fact that neoplasms in the opened testis had in

several cases grown through the incision in the tunic; furthermore, these growths were sometimes larger than those in the opposite testis. That is to say, in thirty-nine rats where grafts succeeded in both testes, a distinctly larger tumor was found in the opened gland in twelve, while in only six was the nodule on this side smaller than that in the intact organ; in twenty-one rats, the intratesticular growths were of uniform size.



TEXT-FIG. 5. $\frac{FRC}{14V}$. Average weight of rats, 78 gm.

The absence of metastases in the abdominal cavity supports the observation of Jones and Rous,[5] that the peritoneum, in animals at least, is not susceptible to metastatic attack unless the subendothelial connective tissues have been injured.

The perfect receptivity of the various organs for inoculated tumors throws some light upon the rationale of metastasis in man. The

[5] Jones, F. S., and Rous, P., *Jour. Exper. Med.*, 1914, xx 404.

belief so long held, that the spleen, for example, actively antagonizes the growth of secondary neoplasms, has been proved untenable by the observation that tumor cells are well able to proliferate in this organ, among the lower animals at least, provided only that they succeed in reaching it. There is no reason to suppose that the human spleen enjoys a higher immunity; indeed, two authors (Kettle[6] and von

TEXT-FIG. 6. $\frac{FRC}{130}$. The rats in this experiment were not weighed.

Hansemann[7]) have but recently asserted that the resistance offered by this organ in man is not nearly so efficient as has been supposed. It is highly probable, therefore, that such exemption as it does possess

[6] Kettle, E. H., *Jour. Path. and Bacteriol.*, 1912–13, xvii, 40.
[7] von Hansemann, D., *Deutsch. med. Wchnschr.*, 1915, xli, 633.

is to be ascribed, not to the exhibition of an antagonism to the tumor cell higher than that opposed by other organs of the body, but chiefly to some mechanical factor. Such an explanation has been advanced recently by Kettle, who suggested that the contractions of the spleen force tumor emboli out of the organ again, and even prevent the growth of a large number of those that have become impacted in the capillaries.

In comparing natural metastasis with artificial metastasis, that is, with inoculation into the various organs, one distinction must not be overlooked; in the former, the tumor cells come to rest in a blood- or a lymph-channel, while in the latter process they are deposited by the needle directly in the tissues. Here they are under more favorable conditions for growth than they would be in the vessel, for a large proportion of natural emboli, unable to establish vascular connections with the vessel wall, perish in consequence, as Schmidt[8] has shown.

The work of Takahashi[9] upon artificial pulmonary metastasis, which he brought about by introducing emulsions of transplantable mouse tumors into the circulation, proves that the cells of certain of these neoplasms are unable to furnish themselves with a stroma from the vessel wall. Belonging, in general, to tumors of slow growth, they die before they have had time to effect the necessary union.

As in the experiments of Weil,[10] the tumor cells did not pass through the lungs, except in one instance. One sarcoma among the fourteen tumors employed by Takahashi (eleven carcinomata and three sarcomata) having done so, seemed to show some predilection for certain sites, resembling in this latter respect carcinomata of the prostate and the thyroid in man, which are especially apt to involve the skeletal system. Whether chemical peculiarities have any part in determining such a selective metastasis is still a disputed question; at present, however, the mechanical influences emphasized by von Recklinghausen and his pupils certainly appear to play the major rôle.

When the recent investigations of metastasis are recapitulated, it

[8] Schmidt, M. B., Die Verbreitungswege der Karzinome und die Beziehung generalisierter Sarkome zu den leukämischen Neubildungen, Jena, 1903.

[9] Takahashi, M., *Jour. Path. and Bacteriol.*, 1915, xx, 1.

[10] Weil, R., *Jour. Med. Research*, 1913, xxviii, 497.

appears that the process is governed by several factors. If a secondary tumor is to be produced, the cells of the primary neoplasm must have the power to establish vascular connections; secondly, they must remain undisturbed in the vessel long enough for this process to be completed. Thirdly, if these two conditions have been fulfilled, the organ in which they happen to lie is probably a matter of indifference in the large majority of cases.

CONCLUSIONS.

The Flexner-Jobling adenocarcinoma of the rat is easily transplantable into the testis of this animal.

The resulting growths in the intact testis do not often attain the size of subcutaneous tumors.

The smaller size of intratesticular nodules can not be explained solely by the pressure to which they are subjected during their growth; other factors, which cannot be determined, appear to be concerned.

EXPLANATION OF PLATE 26.

FIG. 1. Five day graft of the Flexner-Jobling tumor in the testis. Tumor below; testis above. Two capillaries are shown, one cut longitudinally, the other transversely. The old stroma in this field has disappeared, and its place has been taken by fibroblasts from the host. The condition is identical with that of subcutaneous grafts at the fifth day. Magnified 388 diameters.

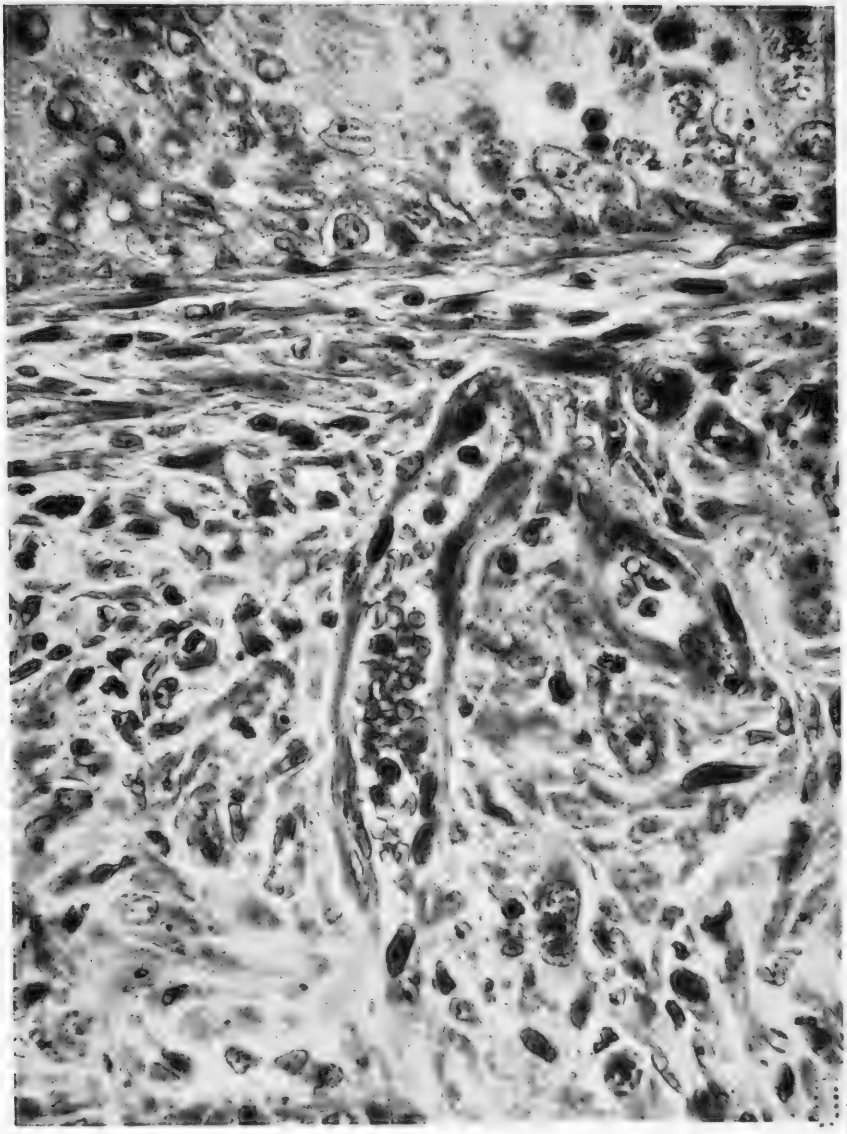


FIG. 1.

(Woglom: Flexner-Jobling Rat Carcinoma.)

EXPERIMENTAL SYPHILIS IN THE RABBIT PRODUCED BY THE BRAIN SUBSTANCE OF THE LIVING PARETIC.

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(Received for publication, November 8, 1915.)

In 1913 Forster and Tomasczewski[1] reported a method for demonstrating *Spirochæta pallida* in the living brain of patients suffering from general paresis. Their method, embodying a modification of the Neisser-Pollak trephining operation, was described by me in 1913.[2]

Forster and Tomasczewski inoculated rabbits with the cortex thus obtained, but their results were negative. In 1913 Noguchi[3] reported two successful inoculations of thirty-six rabbits inoculated with material obtained at autopsy from paretic brains. In the first case, after 97 days, typical nodules appeared in the testes and the scrotal skin. In the second, 102 days elapsed before the nodules appeared. In one case there were extremely few spirochætes, but a large number were demonstrable in the second. An attempt at inoculation into a second generation of the rabbits resulted in a very small lesion. This also appeared only after three months' time.

Berger[4] reported very few spirochætes in the lesions of three out of twenty inoculations, and he made no attempt to prolong the strain. The small number of organisms found in his case makes it probable that these were survivals.

Nichols and Hough[5] reported sixteen inoculations of brain substance from eight cases with slight success in two instances. In their cases, however, small testicular lesions resulted, with striking eye manifestations, but no spirochætes were demonstrable in the lesions, nor were they able to reinoculate other rabbits from the lesions.

All the experiments mentioned above, except those of Forster and Tomasczewski, were made from material recovered at autopsy soon after death.

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[1] Forster, E., and Tomasczewski, E., *Deutsch. med. Wchnschr.*, 1913, xxxix, 1237.

[2] Wile, U. J., *Jour. Am. Med. Assn.*, 1913, lxi, 866.

[3] Noguchi, H., *München. med. Wchnschr.*, 1913, lx, 2484.

[4] Berger, H., *München. med. Wchnschr.*, 1913, lx, 1921.

[5] Nichols, H. J., and Hough, W. H., *Jour. Am. Med. Assn.*, 1913, lxi, 120.

During the past year I have repeated the inoculation experiments of Forster and Tomasczewski, using living material for the inoculation. It seemed to me that the lack of success of previous observers might be due to the small number of organisms present, and I therefore inoculated the brain substance of several cases into a single rabbit. Moreover, it seemed possible that the organisms from a living subject would be more likely to infect than those taken at autopsy.

For my series of experiments six cases were chosen from a large number of paretics. The diagnosis from the clinical findings and from the spinal fluid was frank general paralysis. All the cases had been under the observation of Dr. Edmund A. Christian at the Pontiac State Hospital for a long time, and in each case there were marked manifestations of advanced paresis.

The material was obtained from the brain. The site was prepared with tincture of iodine, with ethyl chloride as a local anesthetic. The skull was trephined over the frontal convolution at a point about one-half to one inch from the midline and well forward of the course of the middle meningeal artery. By means of a long thin trocar needle connected to a syringe a small cylinder of gray and white matter with some fluid from the ventricle was removed. The material was transferred to a sterile Petri dish, containing a few drops of normal salt solution and was at once examined for spirochætes under the dark-field microscope. In five of the six cases spirochætes were demonstrable; in one case they were extremely numerous, and in the remaining four it required from ten minutes' to half an hour's search to demonstrate them.

The major portion of the material thus obtained was at once injected into the testes of a large rabbit (June 11, 1915). The material from five cases was injected into the left organ and that from one case into the right. About two weeks after the inoculation, small hard nodules could be felt in both organs. Aspiration of the nodules after four weeks (July 10, 1915) showed large numbers of active, motile spirochætes. These continued to be demonstrable up to the eleventh week after inoculation, when the animal died from an accidental trypanosome infection, the nature of which was not determined. When the organisms were first demonstrable a second rabbit was inoculated (July 11, 1915) from the aspirated testicular juice of

the first animal. On August 1, 1915, twenty-one days later, both testes were found to be the sites of hard nodules, from which again, on aspiration, large numbers of spirochætes were demonstrable. These continued to be demonstrable in the second rabbit for five weeks, up to the time of the death of this animal from the same accidental infection. A third rabbit was inoculated (August 2, 1915) with the aspirated testicular juice of the second, and fifteen days after inoculation (August 17, 1915) small nodules appeared in both testes, in which again large numbers of organisms could be found on repeated examinations.

Thirty-three days after spirochætes were found in the first rabbit (August 12, 1915), the testis of one side was castrated. The organ was cut into small pieces and a mash made with normal saline solution. A portion of this material was used for cultural work and a small portion for inoculation into a fourth rabbit. At the present writing (October 28, 1915) viable organisms are still present in the cultures made from this mash. Rabbit 4 is still alive (October 28, 1915), and although no nodes are palpable in the testes, *pallidæ* in moderate numbers are still demonstrable by aspiration from both organs.

The work of Noguchi,[3] Nichols and Hough,[5] and Uhlenhuth and Mulzer,[6] seems to indicate the existence of a neurotropic strain of spirochætes. Slight differences in morphology are ascribed to this strain. According to Nichols,[7] the organism is thicker than is ordinarily found, and the curves are not so deep as in the finer variety, the organism being almost as coarse as *Spirochæta refringens*.

The spirochætes in these experiments differ in morphology from those ordinarily seen in mucous and cutaneous lesions. They were similar to those described by Nichols, being shorter and thicker. In all cases, moreover, they seemed to be less actively motile. This may have been partly due to the physical properties of the fluid in which they were studied.

Nichols and Hough reported in their inoculations the occurrence of keratitis and choroiditis from the testicular inoculations. These

[6] Uhlenhuth and Mulzer, *Centralbl. f. Bakteriol., Ite Abt., Ref.*, 1913, lvii, Supplement, 162.

[7] Nichols, H. J., *Jour. Exper. Med.*, 1914, xix, 362.

lesions were identical with those found in acquired syphilis, but the most careful search on their part failed to demonstrate the spirochætes in their cases. None of the rabbits inoculated by me showed either the development of keratitis or other eye symptoms. The rabbit at present living, however, the fourth one inoculated, shows a patchy alopecia, particularly of the head and neck, which is strikingly like that seen in early syphilis.

Noguchi's inoculations required 97 to 102 days of incubation before the development of lesions. His inoculations in the second generation required three months before development. He concludes, therefore, that the infectiousness of general paresis for rabbits is weak and that the virulence of the spirochæte from this source to rabbits is also weak.

In my experiments the first rabbit to be inoculated was found to be infected four weeks from the time of the inoculation and undoubtedly would have proven positive before this time, as nodules were discovered two weeks earlier. The second and third generation showed an increasing virulence as exhibited by the larger numbers of organisms and by the incubation period of three weeks and fifteen days, respectively.

I desire to express my appreciation to Dr. Frederick Novy and Mr. Paul de Kruif for many laboratory courtesies extended to me, and to Dr. Edmund A. Christian for the facilities that he placed at my disposal.

CONCLUSIONS.

1. Spirochætes from the living paretic brain easily infect rabbits with experimental syphilis.

2. They constitute a virulent strain with a shorter period of incubation for the rabbit than exists with other strains.

A STUDY OF THE GASTRIC ULCERS FOLLOWING REMOVAL OF THE ADRENALS.

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PLATES 27 AND 28.

(Received for publication, November 15, 1915.)

It was noted at autopsy that animals dying after the removal of both adrenals showed acute ulceration of the gastric mucosa in a large number of cases.

Cioffi and Pende made this same observation, but Gibelli[1] was the first to give it special attention. The most extensive investigation of the subject was made by Finzi[2] in a study of the gastric mucosa of rabbits and dogs after removal of the adrenals. He found in the gastric mucosa marked circulatory changes consisting of edema, hemorrhage, necrosis, and ulceration. In the few instances in which there was a tendency to heal, the slight healing process involved only the connective tissue, never the epithelium. He found edema and hemorrhagic points in the stomach as early as one hour after extirpation of the adrenals. Dissection of the capsule of the adrenal, leaving the gland intact, did not produce ulceration. The removal of one adrenal in a rabbit produced slight and transient circulatory changes in the gastric mucosa. Animals which were given adrenalin after removal of the adrenals had normal gastric mucosa. In five cases of gastric or duodenal ulcer in man he found microscopic changes in the adrenals, consisting of thickening of the capsule, nodular hypertrophy, fatty degeneration, great congestion, and multiple hemorrhage. He believes that adrenal insufficiency may be a factor in the etiology of gastric ulcer.

Durante[3] investigated the effects of section of the splanchnic nerves on the

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[1] Gibelli, C., La funzione delle capsule surrenali in rapporto col processo di riparazione delle fratture e coll' eziologia dell'ulcera gastrica, *Pathologica*, 1909, i, 131.

[2] Finzi, O., Über Veränderungen der Magenschleimhaut bei Tieren nach Nebennierenextirpation und über experimentell erzeugte Magengeschwüre, *Virchows Arch. f. path. Anat.*, 1913, ccxiv, 413.

[3] Durante, L., Contributo alla fisiopatologia del nervo splancnico, *Pathologica*, 1912–13, v, 631.

gastric mucosa and adrenals in dogs and rabbits. He found that section of all three splanchnics on either side produced no change except congestion in the adrenals; section of the major splanchnic affected the gastric mucosa very slightly; section of either the median or minor splanchnic on either side always produced necrosis and ulceration of the gastric mucosa. The ulcers produced by section of the nerves on the right always healed rapidly and the spleen remained normal; section of the nerves on the left produced changes in the spleen and ulcers in the stomach which tended to become chronic. He attributed the formation of ulcers after adrenalectomy to incidental injury of the minor splanchnic nerves which are buried in the posterior leaf of the adrenal.

Since this investigation was undertaken two articles bearing on the subject have appeared. Elliott[4] notes the frequent occurrence of gastric ulcer on removal of the adrenals in cats, and cites it as proof of the full digestive power of the gastric juice. He refers to the work of Finzi and concludes with the statement that gastric ulcer is not found in Addison's disease.

Friedman[5] investigated the effect on the gastric and duodenal mucosa of removal of the adrenals and one-side thyroidectomy in dogs and rabbits. He considered gastric lesions possibly dependent upon adrenal insufficiency as well as upon an excess of thyroid; duodenal lesions upon hypofunction as well as upon excess of adrenalin; coexistent gastric and duodenal lesions upon alternating conditions of hypo- and hyperfunction of the adrenals. He relies upon the pluriglandular hypothesis of an antagonistic action between the adrenals and the thyroid to explain the formation of gastric and duodenal ulcers and erosions after interference with these glands.

Dogs and cats were used in my investigation.[6] As no lesion of the gastric mucosa was found at autopsy in a series of more than 200 practically normal animals, it would seem that spontaneous ulcers are not common in these animals. Their occurrence after adrenalectomy was studied in the following series of experiments.

Experiment 1.—In four dogs the adrenals were removed at one operation and the animals kept under an anesthetic until death occurred, two or three hours

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[4] Elliott, T. R., Some Results of Excision of the Adrenal Glands, *Jour. Physiol.*, 1915, xlix, 38.

[5] Friedman, G. A., The Influence of Removal of the Adrenals and One-Sided Thyroidectomy upon the Gastric and Duodenal Mucosa; the Experimental Production of Lesions, Erosions, and Acute Ulcers, *Jour. Med. Research*, 1915, xxxii, 287.

[6] These ulcers have also been noted in gophers dying from adrenal insufficiency, and in one case of Addison's disease.

after the removal of the last gland. In these experiments the mucosa of the stomach and duodenum was found to be normal.

Experiment 2.—In twelve dogs the adrenals were removed at two operations. When the second gland was removed the dogs were kept under ether until death occurred, which was from two to eight hours after operation. In none of these animals were gastric or duodenal lesions noted.

Experiment 3.—Forty dogs and six cats were subjected to the removal of one adrenal, usually the right, and killed at periods varying from 5 hours to 235 days after operation. In this series of animals no lesion of the gastric or duodenal mucosa was found except in one dog. This animal died from an unknown cause 4 months after removal of the right adrenal; several acute ulcers and one chronic ulcer were found in the stomach and one acute ulcer in the duodenum.

Experiment 4.—Of sixty dogs and five cats in which both adrenals were removed at the same operation or at different operations, forty animals showed lesions of the stomach, five of these ulcers of both stomach and duodenum. Of the twenty-five animals in which ulcers were not found, only four died an uncomplicated death from adrenal insufficiency; the remaining twenty-one animals were either subjected to other experiments which may have interfered with the formation of ulcers or they died before the ulcer could form.

It is seen that lesions of the gastric and duodenal mucosa did not occur in adrenalectomized animals subject to continuous etherization and were infrequent in animals subjected to the removal of only one adrenal; but lesions in the stomach and duodenum occurred in about 90 per cent of the animals dying with the characteristic symptoms of adrenal insufficiency after removal of both glands. This last fact appears significant in the study of the general causative factors of acute gastric ulcers.

It has been impossible to determine definitely the time necessary for the formation of the ulcers or how soon after complete adrenalectomy they begin to form. In only one of the animals examined within ten hours after removal of both adrenals were any changes noted in the gastric mucosa. In this instance there were several hemorrhagic areas which were possibly the beginning of ulcers. In one animal dying twenty-two hours after extirpation of both glands well formed ulcers were present. In animals examined when muscular weakness was first in evidence, beginning ulceration was noted. No changes in the gastric mucosa were found before decrease in blood pressure took place. It would appear that ulcer formation begins before the onset of the characteristic symptoms of adrenal insuffi-

ciency which progress until the death of the animal, and that only a few hours are necessary for their production.

The lesions found in the gastric mucosa after death from adrenal insufficiency consist of two main types: one is a wide-spread, superficial erosion; the other is a true, punched-out ulcer formation.

The gastric erosions practically always occurred in the fundic division, and in most cases the pyloric mucosa appeared normal. They appeared to begin in and spread along the rugæ, thus producing an irregular appearance. Only the surface of the mucosa was affected, the loss of epithelium never extending to the submucosa. The denuded surface was hemorrhagic in appearance, and the fluid in the stomach was usually blood-stained. This condition developed mainly in those animals in which there was a prolonged moribund condition following the development of muscular weakness.

The gastric ulcers were round or oval in shape and varied in size from 2 mm. to 2 cm. in diameter. They were usually multiple, but a few stomachs contained only one ulcer. Their position varied; they were found in the fundic and pyloric regions on both the greater and lesser curvatures. Usually they occurred in the prepyloric division. Beginning ulcers appeared as small hemorrhagic areas; when fully formed, however, they penetrated to the muscularis mucosa with a complete loss of epithelium. The walls were smooth, giving the characteristic punched-out appearance. A small blood vessel was usually found in the base of the ulcer. When the autopsy was performed immediately after death, in many instances the vessels were bleeding. In the pyloric region the blood gave the ulcer a black appearance, while in the fundus it often remained bright red at the site of the ulcer. In most instances the ulcers constituted the only pathologic change in the mucosa, while in other specimens the mucosa was injected throughout (Figs. 1 and 2).

The duodenal mucosa was usually congested in the adrenalectomized animals. In five experiments there were definite ulcers. These duodenal ulcers occurred just distal to the pyloric ring and appeared like cauterized areas about 1.5 cm. in diameter. They were deeper at the center than at the edges. They penetrated to the muscularis mucosa at the center. They never showed evidence of hemorrhage (Fig. 2).

Microscopically, the picture of the ulcer varies slightly; usually, however, they are cone-shaped with the base of the cone at the surface and the apex at the muscularis mucosa. The edges of the ulcer are clean, although occasionally there may be some cellular débris and blood at the base (Fig. 3). None but the earliest signs of healing have ever been observed.

The loss of tissue appears to begin at the surface. The gland cells disappear first, allowing the supporting tissue to fall together. In some cases the ulcer has not extended to the muscularis mucosa. In these ulcers the gland cells below the base may appear perfectly normal. Hemorrhage seems to be of early occurrence in the formation of the ulcer. In practically all ulcers the blood vessels in the vicinity are congested. The special stains for mucin demonstrate the fact that this substance is usually absent near the ulcers (Fig. 4). The glands in the vicinity of the ulcer may appear normal, but do not contain mucin. The zymogen content of all the cells is decreased, but no more so in the cells around the ulcer than elsewhere.

It has been suggested that the regurgitation of pancreatic secretion is the cause of gastric ulcers and that tryptic ulcer would be a more exact term than peptic ulcer. That the pancreatic secretion is not necessary for the formation of these ulcers was proved by a series of five experiments in which the pancreatic ducts were either doubly ligated and sectioned, or a pancreatic fistula was made before the removal of the last adrenal. In these animals no pancreatic secretion could reach the gastro-intestinal tract, yet many characteristic gastric ulcers were found after death.

It has been demonstrated that bile in association with a strongly acid gastric juice has an erosive action on the gastric mucosa.[7] That the bile might be of importance in the production of these ulcers seemed possible because of the fact that the fluid found in the stomach was usually bile-stained. In one animal the common bile duct was transplanted to the skin, thus making it impossible for any bile to enter the stomach. After complete recovery from the operation,

[7] Smith, G. M., An Experimental Study of the Relation of Bile to Ulceration of the Mucous Membrane of the Stomach, *Jour. Med. Research*, 1914, xxx, 147.

the adrenals were removed. Well formed ulcers were found at autopsy.

The gastric content was always acid in the adrenalectomized animals. In order to determine the part the acid played in the production of the ulcers, an attempt was made to neutralize it during the moribund period. To accomplish this sodium bicarbonate was administered about every four hours, either in solution by stomach tube or in capsules. Of course, it was impossible to be sure that the gastric contents were always kept neutralized, but certainly in most instances no great excess of acidity developed. In a series of ten experiments in which sodium bicarbonate was administered after the removal of the last adrenal, ulcers were found in one animal only. In this experiment it is possible that the bicarbonate was not given frequently enough to prevent the development of acidity. This result would tend to show, as has been demonstrated in regard to the formation of other acute ulcers, that acid is a factor in their production.[8]

In a series of ten experiments a gastro-enterostomy was performed during the interval between the removal of the adrenals. In only four of these animals were ulcers found. It is possible to explain this result as due to a reflux of the alkaline intestinal secretion and the bile.

SUMMARY.

Acute ulcers of the gastric mucosa are found in a large percentage of dogs and cats dying after adrenalectomy. These ulcers seem to develop during the moribund period. They are apparently peptic ulcers forming at the site of local hemorrhages in the gastric mucosa. They are true acute ulcers, usually penetrating to the muscularis mucosa with a total loss of epithelium. They develop in the absence of pancreatic secretion and bile. However, they appear to develop only in an acid medium.

[8] Bolton, C., Ulcer of the Stomach, London, 1913, 59.

FIG. 1.

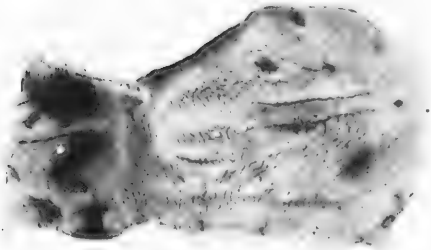

FIG. 2.

FIG. 3.

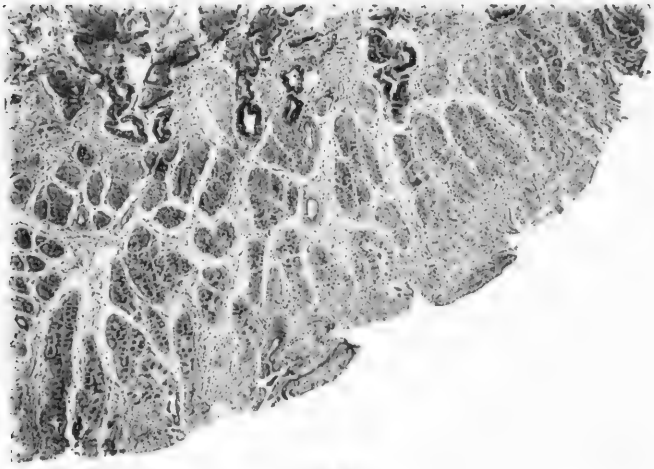

FIG. 4.

(Mann: Gastric Ulcers.)

EXPLANATION OF PLATES.

PLATE 27.

FIG. 1. Gastric mucosa of a dog, showing multiple acute ulcers.

FIG. 2. Pyloric and duodenal mucosa, showing multiple acute ulcers.

PLATE 28.

FIG. 3. The center of a gastric ulcer, showing the clean edges and base.

FIG. 4. The edge of a duodenal ulcer, showing the loss of goblet cells (the dark stained cells) in the vicinity of the ulcer.

A TRANSPLANTABLE CARCINOMA OF THE GUINEA PIG.

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PLATES 29 TO 32.

(Received for publication, September 15, 1915.)

The successful transplantation of rat and mouse tumors has led to many attempts to transfer the tumors of other species. The venereal lymphosarcoma of dogs, transferred by coitus, has been studied by a number of investigators, especially Sticker.[1] Von Dungern and Coca[2] described, and succeeded in transferring an epidemic tumor of the hare. They[3] were able to transfer the venereal dog tumor to foxes and the hare tumor to rabbits. The status of these growths must be considered doubtful since it has been well established that neoplasms in general are only transplantable within the species in which they occur spontaneously. Ribbert[4] has described a fibroma of the dog which he inoculated successfully into two other series of dogs.

Rous[5] succeeded in transferring a spindle-celled sarcoma of a fowl. Later he and his collaborators reported a number of other transmissible avian sarcomata. Fujinami and Inamato[6] have transplanted a myxosarcoma of the fowl. More recently Schultze[7] described a spindle-celled sarcoma of the rabbit which he had propagated through twelve tumor generations. In the laboratories of the Imperial Cancer Research Fund of London another rabbit sarcoma has been studied.

Up to the present a transplantable tumor of the guinea pig has not been reported. Few data are obtainable concerning the tumors of this species; apparently they are rare. Sternberg[8] reported a metastasizing adenocarcinoma of the mammary gland of the guinea pig. He did not attempt transplantation.

[1] Sticker, A., *Ztschr. f. Krebsforsch.*, 1904, i, 413.
[2] von Dungern and Coca, *Ztschr. f. Immunitätsforsch., Orig.*, 1909, ii, 391.
[3] von Dungern, *München. med. Wchnschr.*, 1912, lix, 238.
[4] Ribbert, H., *Centralbl. f. allg. Path. u. path. Anat.*, 1910, xxi, 625.
[5] Rous, P., *Jour. Exper. Med.*, 1910, xii, 696.
[6] Fujinami, A., and Inamato, K., *Verhandl. d. jap. path. Gesellsch.*, 1911, i, 114.
[7] Schultze, W. H., *Verhandl. d. deutsch. path. Gesellsch.*, 1913, xvi, 358.
[8] Sternberg, C., *Verhandl. d. deutsch. path. Gesellsch.*, 1913, xvi, 362.

The tumor which I have transplanted occurred in the mammary gland of an old female guinea pig which, judging from the condition of the breast, had recently suckled offspring. This animal was removed from the Institute breeding pens, with a number of others suffering with abscesses. It had a large fluctuating abscess in the submental region and a large, rather firm, sharply circumscribed tumor in the left mammary gland. This tumor lay in the substance of the gland directly beneath the skin, but was not attached to the subcutaneous tissue or musculature. It was hemispherical in shape, of a grayish white color, and measured 4.5 by 4 by 3.6 cm. The mass was enveloped in a delicate, translucent capsule. It was removed and cut across. Just beneath the capsule was a zone of grayish white, translucent tissue 0.5 to 1 cm. wide. A milky fluid exuded from its cut surface. Toward the center of the tumor this zone gradually faded off into a rather granular necrotic material. The color of this portion of the growth varied from yellowish white to ocher. The diagnosis of cancer was made from frozen sections of the outer zone of grayish white tissue. Examinations of the regional lymph glands and other organs for metastases were negative.

Bits of the peripheral layer of the tumor were inoculated by means of a trocar into the subcutaneous tissue of the groin and into the muscles of the upper leg of sixteen young guinea pigs. Most of the animals were between two and three weeks old. Animals of this age were chosen because it has been frequently shown that young individuals are especially favorable hosts for tumor grafts.

First Tumor Generation.

Oct. 30, 1914. Sixteen young guinea pigs were inoculated with two small pieces of the tumor in the subcutaneous tissue of the left groin and the muscles of the left upper leg. The pieces varied from 0.15 to 0.2 cm. in diameter.

On Jan. 15, 1915, one of the animals showed a swelling in the muscles of the leg at the site of inoculation (Fig. 1). The growth was irregularly rounded and protruded sharply from the surface of the leg. It measured 2.1 by 1.7 by 1.2 cm. The subcutaneous graft in the left groin could not be detected on palpation.

On Jan. 20, 1915, the animal was anesthetized with ether and the major portion of the tumor removed. It lay within the muscles, protruding sharply from the general contour of the leg. Externally it was covered with a thin sheet of muscle. The mass was encapsulated on its external surface with a thin, trans-

lucent layer of connective tissue. The inner surfaces were firmly fixed to the muscles. Nearly all of the tumor was removed in small pieces. These consisted of pink, friable tissue, some portions of which were hemorrhagic. A few circumscribed areas of necrosis were noted. The incision in the skin was closed with silk. Most of the tissue was used to inoculate another series of animals. The remainder was fixed in Zenker's fluid for microscopic study.

The other animals of this series were under observation for over 6 months. None of them developed tumors.

Histology of the Primary Tumor.

Sections of the original tumor show it to be a carcinoma. The cells are arranged in acini, or more or less irregular clumps, supported by a delicate connective tissue stroma. The proliferating portion is confined to a narrow layer surrounded by a thin capsule. In one section the cancer cells have penetrated this capsule and invaded the mammary gland (Fig. 5). The individual cells in the growing portion are large, with a large vesicular nucleus. They stain well. A few mitotic figures are seen. Toward the center the cells appear shrunken and the nuclei stain intensely, while still further in all cell structure is lost. The tumor is well vascularized, but not hemorrhagic.

A few eosinophils are found along the borders of the tumor. In several places its cells have invaded the fatty tissue of the gland (Fig. 5). The interacinar connective tissue is increased throughout the section. Many of the neighboring acini and ducts show pressure changes. They are flattened and the epithelium is atrophied. The wall of one of the larger ducts adjacent to the tumor has become infiltrated with tumor cells and the growing tissue extends as a considerable mass into the duct lumen. There is a marked collateral hypertrophy of the lining epithelium and the interacinar mass is partly covered by a double layer of non-malignant columnar cells (Fig. 7).

Of great interest are certain changes in the breast that cannot be attributed directly to the tumor. One finds the atrophy, overgrowth of connective tissue, and chronic inflammatory changes seen in the breasts of old animals of other species. Some of the acini at considerable distance from the tumor show proliferative changes. Their epithelium has proliferated until they are nearly occluded with a densely packed mass of cells (Fig. 6). Apparently the cells in lesions

of this type show no tendency to break through the acinar walls. A few acini reveal still more advanced changes and have almost lost their character. The remains of such an acinus consist of an irregular clump of cells with an ill defined basement membrane. As a whole, the changes are similar to the so called precancerous lesions noted by Haaland[9] in the breasts of old female cancerous and non-cancerous mice. McCarty[10] has also described the same conditions of the mammary glands of women suffering from carcinoma of the breast.

The Tumor of the First Transplantation.

It has been stated in the protocol that a tumor developed slowly in one animal inoculated with small pieces of the spontaneous tumor (Fig. 1). The greater portion was removed, but it began to recur 3 weeks after operation.

Mar. 3, 1915. The operated tumor remained quiescent for about 3 weeks and then began to grow rapidly. On this date it measured 2.4 by 1.5 by 1.5 cm. The enlarged inguinal lymph gland on the same side was felt as an oval disc measuring 1.9 by 1.4 by 0.6 cm. (Fig. 2). Growth of the subcutaneous graft had not taken place.

Mar. 8, 1915. The animal was found dead. The leg tumor measured 2 by 1.6 by 1.2 cm. It was an irregular flattened sphere with a smooth projecting surface. The deeper portions had invaded the muscle. On section the growth was divided into numerous lobules by strands of connective tissue. Considerable brownish yellow, necrotic material was noted. The peripheral portions of the tumor were composed of friable, translucent, yellowish white tissue which exuded a milky fluid.

The disc-shaped mass in the inguinal region proved to be a metastasis in which the lymph gland had been replaced almost entirely with tumor tissue. It measured 1.2 by 1.1 by 0.6 cm. and it was made up of the same type of tissue as the leg tumor. About 1.5 cm. above the metastasis there were two tiny, discrete, hemispheral, grayish white nodules on the external surface of the abdominal muscle. These were doubtless a part of the tissue supposed to have been inoculated subcutaneously.

On opening the abdominal cavity a large, irregular, ovoid tumor was found. It measured 6.6 by 5.0 by 3.8 cm. and lay on the right side between the liver and

[9] Haaland, M., *Fourth Scientific Report of the Imperial Cancer Research Fund*, 1911, 1.

[10] McCarty, W. C., *Surg., Gynec. and Obst.*, 1913, xvii, 441.

cecum (Fig. 4). Broad bands of connective tissue connected it with the neighboring viscera and peritoneum. Its surface was smooth and glistening. The mass was divided by fissures into four principal lobules which in turn were separated into a large number of small lobulations. The color varied. The sounder portions were grayish white and translucent. Considerable hemorrhage had occurred beneath the capsule, which resulted in a gray and red mottling. The capsule was thin and well vascularized.

On section the surface lobulations were found to extend throughout. The four principal ones were separated by broad bands of connective tissue. At both ends and along the superior border was a layer of translucent, grayish white, nearly homogeneous tissue. The balance of the tumor was practically comprised of pinkish or reddish gray necrotic material.

Other tumors were not found in the abdominal cavity. Gross or macroscopic metastases were not observed in the other organs or glands.

Doubtless the large abdominal tumor was responsible for the death of this animal. From the location and general character of the tumor it seemed evident that it resulted from direct transplantation. Probably the cannula, with which the subcutaneous inoculation had been made, pierced the abdominal wall, and a small portion of the tumor lodged in the mesentery. The inguinal tumor was unquestionably a metastasis from the leg mass to the inguinal lymph gland.

Second Tumor Generation.

Series A.—Jan. 20, 1915. Thirty young guinea pigs were inoculated in the leg muscles and in the subcutaneous tissue of the groin with small pieces of tumor from No. 31.

Feb. 26, 1915. One animal, No. 26, had developed a tiny shot-like nodule in the leg and groin.

Mar. 8, 1915. Animal 26 was found dead. In the muscles of the leg at the site of inoculation were three round, raised, grayish red nodules varying in size from 0.2 to 0.4 cm. In the subcutaneous tissue of the groin 6 small glistening gray nodules were found embedded in the fascia. Microscopically these consisted of living tumor tissue, doubtless that which had been implanted. Metastasis to the other organs had not taken place. Bronchopneumonia caused the animal's death.

Mar. 8, 1915. Two other guinea pigs, Nos. 9 and 38, had developed small tumors in the leg and groin. The greater portion of the leg graft of No. 9 was removed and inoculated into the leg muscles of twenty young animals (3rd Tumor Generation, Series A).

May 1, 1915. The operated tumor recurred very slowly at first, but later grew rapidly. On this date it measured 2 cm. The animal was again anesthetized and a considerable portion of the leg tumor removed; bits of it were injected into twenty-five others (3rd Tumor Generation, Series B).

May 11, 1915. The leg tumor of No. 38 had grown slowly. At this time it measured 1.8 cm. in diameter. The major portion of it was removed, chopped fine, and drawn into a syringe. Five young guinea pigs were inoculated in the leg muscles with small amounts of the hash.

May 15, 1915. The tumor of No. 9 enlarged rapidly after the last operation, but soon began to soften and become a huge, almost spherical swelling. It involved the whole upper leg and hip and extended to the mammary region (Fig. 3). It measured 4.7 by 4.2 by 4.2 cm. In the leg of Guinea Pig 38 a large diffuse wedge-shaped nodule had appeared.

June 18, 1915. Animal 9 was found dead. The leg tumor had begun to ulcerate several days before. At autopsy it was found to be largely necrotic The inguinal lymph glands draining the tumor were enlarged. Gross tumors were not found in other organs. Microscopic examination of the enlarged inguinal lymph glands revealed a metastatic tumor in one of them. Metastatic tumor cells were observed in sections of the kidney. They were scattered in irregular aggregates in the external portion of the medulla. The process seemed to have begun in the interlobular capillaries with consequent invasion and obliteration of the tubules. Metastases were not found in the lungs or other organs.

Series B.—Mar. 8, 1915. Twenty young guinea pigs were inoculated by means of a cannula in the leg muscles and in the peritoneal cavity with bits of the leg and abdominal tumors of No. 31, obtained some hours after death. The animals were under observation over 4 months, but none of them developed a tumor.

Third Tumor Generation.

Series A.—Mar. 30, 1915. Twenty young guinea pigs were inoculated with cannulas in the left leg with bits of tumor from No. 9.

Apr. 16, 1915. No. 103 had a small tumor at the point of inoculation.

May 11, 1915. Three animals, Nos. 103, 77, and 67, had developed tumors of a rather uniform size in the inoculated leg muscles. The largest measured 2.3 cm. and the smallest 1.8 cm. On operation most of the tumor of No. 103 was necrotic. Five young guinea pigs were inoculated in the leg with the better portions of the growth (4th Tumor Generation, Series A). Twenty others were injected in the leg with small pieces of tumor incised from the leg of No. 77 (4th Tumor Generation, Series B).

June 16, 1915. The tumor of No. 67 was incised and several small portions of it were inoculated into the upper leg of 15 young guinea pigs (4th Tumor Generation, Series C).

The Transplanted Tumor.

There has been a gradual increase in the number of takes. In the later generations the tumors have appeared earlier and have grown more rapidly and reached a larger size.

Microscopically the transplanted tumor consists of a zone of variable width of rapidly proliferating epithelial cells surrounded by a thin capsule. The cells may be grouped in acini, but they usually lie in small irregular clumps. Large numbers of them may be undergoing mitotic division (Fig. 9). Degenerative changes similar to those observed in the spontaneous tumor are frequent. At the growing edge small blood vessels are numerous. Occasionally they have the form of thin-walled sinuses (Fig. 8). One is struck by the slight reaction about the tumors; practically no round celled infiltration is observed about their borders. The connective tissue capsule is well defined but thin.

SUMMARY.

An adenocarcinoma of the mammary gland of an old guinea pig has been successfully transplanted through eight successive series of animals. It now appears much earlier and grows more rapidly. The number of takes also has increased. In two instances metastasis to the regional lymph glands (inguinal) has been observed. Once microscopic metastases were found in the kidney. The so called precancerous changes observed in the breasts of women and mice suffering from mammary carcinoma were found in the mammary gland of the spontaneous tumor animal. It is hoped that the tumor may soon be utilized for experimental purposes.

EXPLANATION OF PLATES.

PLATE 29.

FIG. 1. The tumor in the leg of Guinea Pig 31 of the 1st Tumor Generation, 81 days after inoculation.

FIG. 2. Recurrence in the leg of the same animal, at autopsy 47 days after partial removal. Note the metastasis to the inguinal lymph gland.

FIG. 3. Large cystic tumor of the leg of Animal 9 of the 2nd Tumor Generation, 4 months after inoculation. On two different occasions large portions of the growth had been removed.

PLATE 30.

FIG. 4. The abdominal tumor found at autopsy of No. 31 of the 1st Tumor Generation. It has been reflected to show the bands of attachment.

PLATE 31.

FIG. 5. Cells of the spontaneous mammary tumor invading the fatty tissue of the gland.

FIG. 6. Epithelial inclusions in the lumen of the mammary acini of the original animal.

FIG. 7. Growth of the cells of the spontaneous tumor through the wall and into the lumen of one of the larger milk ducts. The growth of the cancerous epithelium has been accompanied by a collateral growth of the lining columnar cells. Note the pressure changes of the smaller ducts.

PLATE 32.

FIG. 8. The characteristic border of growing neoplastic cells with many blood sinuses in a section of transplanted tumor.

FIG. 9. The transplanted tumor in a later generation showing the usual arrangement of cells. Many of them are undergoing mitosis.

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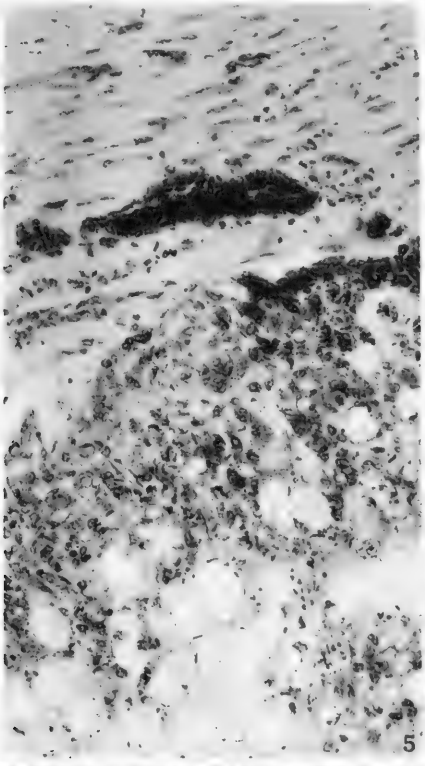
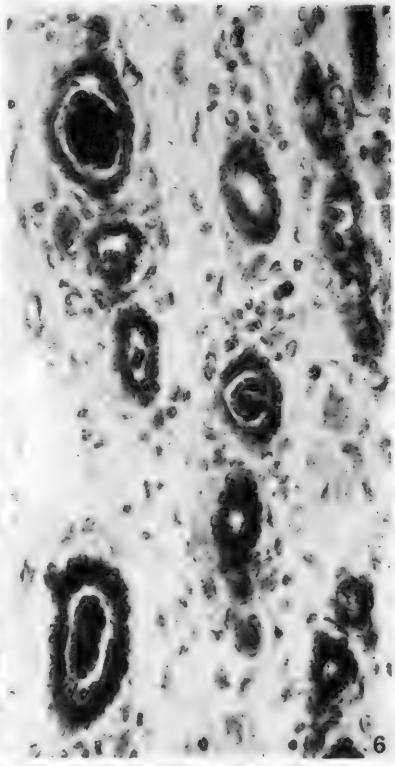
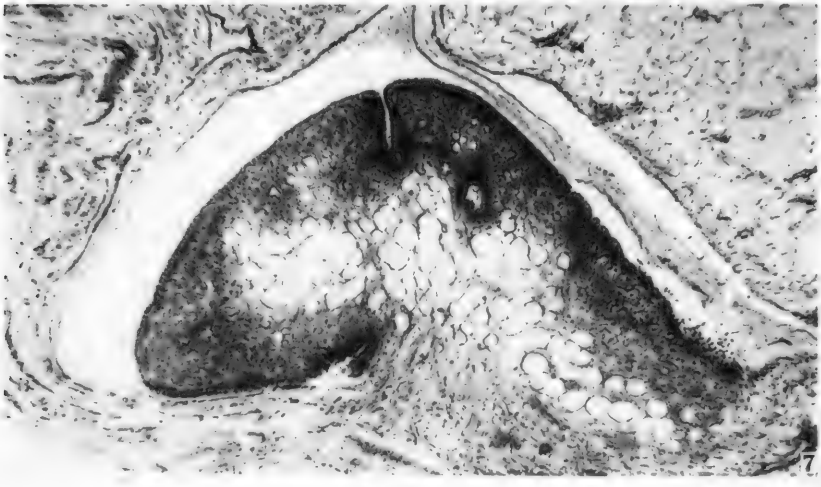
(Jones: Transplantable Carcinoma of Guinea Pig.)

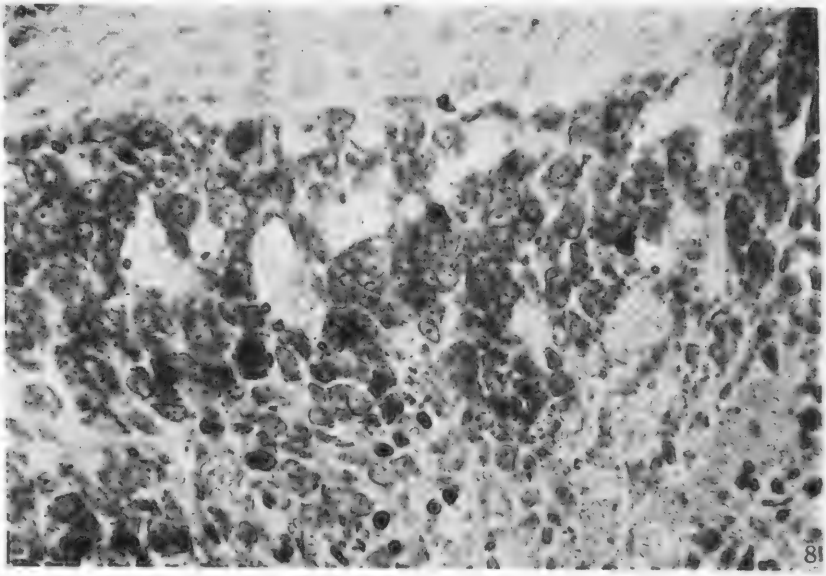
(Jones: Transplantable Carcinoma of Guinea Pig.)

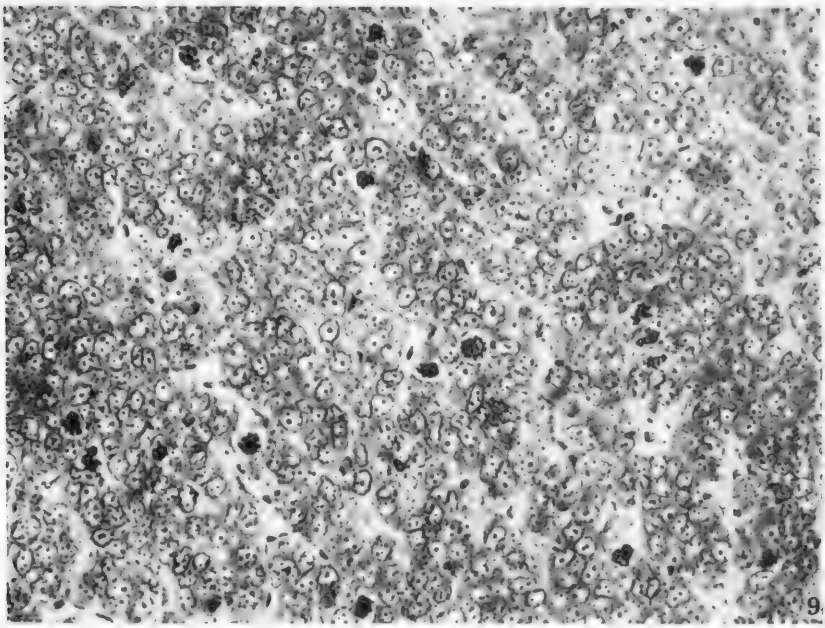






(Jones: Transplantable Carcinoma of Guinea Pig.)





(Jones: Transplantable Carcinoma of Guinea Pig.)

THE PRESERVATION OF LIVING RED BLOOD CELLS IN VITRO.

I. Methods of Preservation.

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There is practically no mention in the literature of attempts to keep red blood cells alive for a long time *in vitro*. Yet methods for their preservation might have much practical importance, and certainly would possess theoretical interest. Kept cells could be utilized for serum reactions, or for culture media, or even under certain circumstances for transfusion. This last possibility is the more worth considering because of the recent experiments of Abel and his coworkers[1] on plasmaphaeresis. They have demonstrated that the body tolerates well the repeated withdrawal of large amounts of plasma, rapidly placing new fluid in circulation; from which it follows that only the formed elements of blood need be supplied to a healthy animal depleted by hemorrhage.

The essential peculiarities of the red blood cells must be taken into account in any attempt to preserve them. They have little of the ability to adjust themselves to changes in external conditions which is possessed by many somatic cells[2] in common with the unicellular organisms. As bits of protoplasm without a nucleus, multiplication is impossible to them, and their existence is necessarily limited, whether they are in the circulation or *in vitro*. If they are to be kept alive outside the body, it must be in what one might term a state of suspended

[1] Abel, J. J., Rowntree, L. G., and Turner, B. B., *Jour. Pharmacol. and Exper. Therap.*, 1914, v, 625.

[2] Striking instances of this ability are afforded by the *in vitro* growth of tissue cells in plasma diluted with distilled water, in plasma from other animal species, in synthetic media, etc.

animation. Experience has taught that they are best kept in the cold. But even then whether the medium be serum or physiological salt solution, they break down within a few days,—much sooner than they are supposed to do in the circulation. Evidently there is room here for improvement in method, or else current estimates of the length of life of the red cell are faulty.

Injury during Washing.

For our experiments we have used cells of the rabbit, dog, sheep, and man. It seemed best in the initial tests to free the cells of plasma and suspend them in solutions of known constitution. Meltzer[3] has shown that red cells shaken in plasma to the slight extent necessary for defibrination break down much sooner on keeping than when the blood is allowed to clot undisturbed. We have asked ourselves whether injury during washing might not be responsible for the brief survival of washed cells. That in the case of dog, rabbit, and sheep cells, especially the first mentioned, some injury may occur is evident from the hemolysis frequently observed during washing even when the cells are handled most carefully.

Experiment 1.[4] *Cells Washed and Kept in Ringer's Solution Break Down Sooner than Those Left Undisturbed in Citrated Blood.*—Dog blood was taken in Ringer's solution containing 1 per cent of sodium citrate in the proportion of 1 part of blood to 4 of the solution. Some of the mixture was at once tubed and set aside. The cells of the remainder were washed twice with ordinary Ringer's solution and distributed in Ringer's plus citrate and in Ringer's solution, respectively, to the same amount as in the original blood-citrate mixture. The tip of the pipette with which the washing was carried out was kept beneath the surface of the fluid, and in other ways the handling was careful. There was no immediate hemolysis. Tubing was done as in the case of the unwashed blood. The preparations were kept in the ice box.

Two days later hemolysis was well marked in the preparations of washed blood. The cells of the original citrated blood were still intact.

Experiment 2. Cells Washed and Kept in a Plasma-Locke's-Citrate Mixture Hemolyze Sooner than Those Left Unwashed in the Same Mixture.—Rabbit blood

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[3] Meltzer, S. J., *Rep. Johns Hopkins Hosp.*, 1900, ix, 135.

[4] The experiments are all specimen instances selected from a number giving the same results.

was taken in Locke's[5]-citrate,—1 part of blood to 4 of the solution, which latter contained 1 per cent of citrate,—and some of the mixture was tubed and set aside. The cells of the remainder were washed twice with Locke's fluid and to some of the cells the original supernatant fluid, a mixture of plasma and Locke's-citrate, was restored, in the original proportion, and tubing done as usual.

After five days in the ice box there was well marked hemolysis of the washed red cells but none of those left undisturbed.

Experiment 3. Red Cells That Break Down Rapidly when Shaken in Locke's Solution Remain Intact in Plasma-Locke's-Citrate. The Plasma is the Protective Agent.—Some blood was taken from two normal sheep, two rabbits, two dogs, and two men into Ringer-citrate, in the usual 1: 4 proportion. Half of each mixture was washed twice with Ringer's solution and the original volume restored with it. There was no hemolysis. The washed and unwashed specimens of blood were now placed in shaking tubes of uniform size. The same considerable air space was left in each tube and duplicate preparations were made. All were now shaken in a machine for 15 minutes, the tubes centrifugalized, and the amount of hemolysis read.

The washed human bloods shaken in Ringer's solution showed each a trace of hemolysis, the two sheep bloods considerably more, an amount which may be indicated by +, the rabbit bloods +++, and the dog bloods +++ and ++++, respectively. There was no hemolysis in any of the tubes containing plasma-Ringer's-citrate. The duplicate preparations confirmed these results.

As a corollary to this experiment, tests were made to see whether Ringer's-citrate without plasma had a protective action. Washed cells shaken in it went to pieces with the same rapidity as in ordinary Ringer's solution.

It is evident that the handling of red cells in salt solution, even to the small extent necessary to wash them, may be very injurious. Much of the injury is immediate and mechanical in character. Plasma has a notable influence to prevent it.

Protection.

From these observations it is plain that if washed red cells are to be properly preserved they must be protected during washing. Plasma obviously cannot be used for this purpose. Some simple agent is needed. And this was found in gelatin. $\frac{1}{8}$ to $\frac{1}{4}$ per cent of gelatin in Locke's solution protects cells absolutely against injury during washing, and even during prolonged shaking.

[5] The Locke's solution referred to here and elsewhere is Locke's modification of Ringer's fluid, but without any sugar: 9.2 gm. sodium chloride, 0.05 gm. sodium bicarbonate, 0.1 gm. potassium chloride, 0.1 gm. calcium chloride in 1,000 cc. of water.

Experiment 4. The Protective Influence of Gelatin.—Dog blood was taken in Locke's-citrate as usual (1 part to 4), distributed in equal quantity in eight tubes, and centrifugalized at high speed. From all except one of the tubes the supernatant fluid was now pipetted off as completely as possible, by means of a capillary pipette. The original volume was restored to six tubes with Locke's solution containing graded amounts of gelatin, from $\frac{1}{2}$ per cent to $\frac{1}{64}$ per cent, while to the seventh tube ordinary Locke's solution was added. All were now stoppered, shaken in a machine for 15 minutes, centrifugalized, and the hemolysis was noted. Shaking was then renewed for 15 minutes, and the tubes were placed in the ice box and examined after 3 days. The results are given in Table I.

TABLE I.

Hemolysis.

Time of shaking.	Locke's solution	+ $\frac{1}{64}$ per cent gelatin.	+ $\frac{1}{32}$ per cent gelatin.	+ $\frac{1}{16}$ per cent gelatin.	+ $\frac{1}{8}$ per cent gelatin.	+ $\frac{1}{4}$ per cent gelatin.	+ $\frac{1}{2}$ per cent gelatin.	Citrated blood.
min.								
15.....	+++	+++	+++	+−	0	0	0	0
30.....	+++	+++	+++	+−	0	0	0	0

Many subsequent experiments confirm this one. The protection afforded by the addition of $\frac{1}{8}$ per cent of gelatin to Locke's solution is for practical purposes perfect, and we have employed it regularly when blood was to be washed. Cells of the sheep, dog, and rabbit thus protected last days longer than when handled in ordinary Locke's solution.

Specific Differences in Fragility.

Protection is especially needed in the case of the red cells of the dog. Ottenberg, Kaliski, and Friedman[6] in some experiments on the normal hemagglutinins of this animal found that erythrocytes washed and placed in salt solution broke down too rapidly to be used. They were obliged to employ cells suspended in their own serum. Usually we have noted an abundant hemolysis within a few hours of dog cells washed after the ordinary methods. But if gelatin-Locke's be the washing medium, they may remain intact for several days. The erythrocytes from different dogs show marked differences in their period of survival.

[6] Ottenberg, R., Kaliski, D. J., and Friedman, S. S., *Jour. Med. Research*, 1913, xxviii, 141.

It has proved interesting to compare the resistance to mechanical injury (shaking) of the cells of different species. The results obtained in Experiment 3 express a general rule. Human cells have by far the greatest resistance. In their case, as we have repeatedly found, washing with Locke's solution after the ordinary method entails no perceptible injury, the cells remaining unhemolyzed as long as when gelatin-Locke's is used. Sheep cells come next in point of endurance. But washing in ordinary Locke's solution injures them somewhat, and it affects much more considerably rabbit cells and dog cells. All of these require protection by gelatin. An absolute scale of the fragility of bloods is difficult to prepare because marked variations are observed with the cells of different individuals and with the length of time that shaking is carried on. Dog blood at first breaks down far more than rabbit blood, but as shaking is continued the latter shows the greater destruction. In order properly to illustrate the findings, curves should be constructed like those that Smith and Brown[7] have used to record the percentages of erythrocytes breaking down in salt solutions of graduated hypotonicity.

Resistance to Shaking versus Resistance to Hypotonic Solutions.

Has the resistance of erythrocytes to mechanical injury any relation to their behavior in hypotonic salt solution?

TABLE II.

	Sheep C.	Sheep M. A.	Dog B.	Dog Dal.
Minimum resistance in salt solution=	0.76 per cent.	0.70 per cent.	0.58 per cent.	0.50 per cent.
Hemolysis on shaking.	Tr.	++	++++	++++

Experiment 5. The Resistance of Erythrocytes to Hypotonic Salt Solution, and to Shaking Vary Independently.—Blood was taken from two dogs often bled previously and from two sheep immunized against a bacterium and also bled often. 15 cc. of each blood were allowed to flow into an equal amount of a solution containing 4 per cent of sodium citrate, 0.6 per cent of sodium chloride, and the other salts of Locke's solution in the usual amount.

[7] Smith, T., and Brown, H. R., *Jour. Med. Research*, 1906, xv, 425.

Each specimen was divided into two equal parts; these were washed twice with Locke's fluid, and one was made up with it as a 10 per cent suspension for shaking, while the other, in thick suspension, was used for tests of resistance to salt solution. Shaking was carried on for 10 minutes and the tubes were centrifugalized and read. Two drops of the thick suspension were added to a number of tubes containing 3 cc. of hypotonic sodium chloride solution. The tubes of hypotonic salt solution were so prepared that each differed from the next by 0.02 per cent of sodium chloride. Readings were taken from these after 3 hours and again after 12 hours.

The results are shown in Table II. The percentages of salt solution recorded are those giving the faintest trace of hemolysis.

In this experiment the cells of the sheep were far less resistant to hypotonic salt solution than those of the dogs, but to shaking they exhibited much the greater resistance. The same phenomenon was evident in the case of individuals of one species (Sheep C. and M. A.). This inverse relationship between the two resistances does not always hold. Human erythrocytes, as we have found, are very resistant to shaking and quite resistant to hypotonic solutions. Hamburger[8] has pointed out that resistance to hypotonic salt solution is the result of many factors, and Rywosch[9] and Rous[10] respectively have proved that it is independent of resistance to chemical hemolysins and to a specific serum hemolysin. The fact that it is also independent of resistance to mechanical injury shows that the term fragility, so often used in connection with it, is a misnomer. Resistance to hypotonic solution is in no real sense an index to the fragility of red cells. A clinical investigation of this fragility as determined by shaking experiments might be not without importance.

Protection versus Preservation.

The injury sustained by cells washed and kept in salt solution is one cause for their rapid breaking down, but it is not the only one.

Experiment 6. Locke's Solution Is Injurious to Cells Kept in It after Washing.—Dog blood was taken as usual in citrate and twice washed, part with Locke's

[8] Hamburger, H. J., Osmotischer Druck und Ionenlehre in ihrer Bedeutung für die Physiologie und die Pathologie des Blutes, Berlin, 1912.

[9] Rywosch, D., *Arch. f. Physiol.*, 1906–07, cxvi, 229.

[10] Rous, P., *Jour. Exper. Med.*, 1909, xi, 763.

solution and part with Locke's solution containing ½ per cent of gelatin. The fluid was taken off, as far as possible, by means of a capillary pipette, and the cells were made to 12½ per cent suspension with ordinary Locke's. 1 cc. of each suspension was now added to 3 cc. of the original plasma-Locke's-citrate and to ordinary Locke's solution, respectively. Examination of the tubes after they had been 4 days in the ice box showed that there was no hemolysis of the cells washed in gelatin-Locke's and kept in plasma-Locke's-citrate, and only the faintest trace in the case of those washed in Locke's. Cells kept in Locke's after washing in gelatin-Locke's showed a + hemolysis, and those washed in plain Locke's a ++ hemolysis.

Here the protective action of gelatin is evident. But it is also plain that, whether red cells are protected during washing or not, they break down sooner in Locke's solution than in a mixture of Locke's with plasma and sodium citrate.

Preservation.

The search for a preservative was now begun. The form of the experiments was simple. Blood was taken in Locke's solution containing 1 per cent of sodium citrate, and its cells were twice washed with Locke's containing ⅛ per cent of gelatin. All possible fluid was then pipetted off, the cells made up in suspension with Locke's, and portions added to the various preservative fluids. These had, for the most part, Locke's solution, without sugar, as a base. We are aware that other solutions, Tyrode's for example, might have proved better; but Locke's fluid has the advantage of simplicity, and some preliminary observations suggested that cells last longer in it than in 0.95 per cent sodium chloride or in ordinary Ringer's fluid. The substances tested for preservative action were all of high purity. Small vials with sterile corks were used as containers, and only enough cells were placed in each to cover the bottom thinly. This was done because it was found that cells allowed to sediment in a thick layer exhibit very slowly the action of the supernatant fluid. So many vials were used in each experiment that it was not practicable to test the sterility of each preparation by means of culture, and instead reliance was placed on duplicate preparations, on repetition of the work, and on the graded character of the tests which ensured graded results except when some technical error was present. The preparations were kept in the dark under aseptic conditions at a temperature of 1° to 3°C. At first the

appearance of hemolysis was taken as the limit of the period of survival of the cells; but as the work progressed and more precise evidence was needed, it was obtained by transfusing the kept cells in bulk. The transfusion experiments are dealt with in the second part of this paper.

The action of sodium citrate was tested out because of the results with plasma-Locke's-citrate. But though a 1 per cent solution of it in Locke's fluid proved better for dog and rabbit corpuscles than plain Locke's solution, it had not sufficient preservative action for practical purposes.

The first step toward preservation of the cells seemed to be to obtain conditions that would not be deleterious to them. In salt solutions such as Ringer's and Locke's the conditions are far from natural. Red cells placed in them undergo a change of form, becoming spherical (Hamburger).[11] Weidenreich[12] attributes this to the absence of colloids. So tests were begun with Locke's solution to which colloids of various sorts had been added. Gelatin was first employed. It has no preservative action, but, on the contrary, in the amount of $1\frac{1}{2}$ per cent, which restores the normal shape to the cell (Weidenreich), it causes a gradual hemolysis and browning of the blood pigment. Washed agar, soluble starch, plain starch, and dextrin were also tried, but only the last proved useful, and that only for dog blood. The fluid pressed from serum coagulated by heat, and serum water made up to isotonicity with the salts of Locke's gave poor results.

A number of observers[13] have shown that the red cells are almost totally impermeable to sugars in contrast with other crystalloid substances. Solutions of sugar, then, not inconceivably might act like colloids in their effect on the shape of the cells. Sugars dissolved in Locke's fluid were accordingly tried and in them red cells were found to be preserved intact for a remarkable length of time. Dextrose and saccharose had the most marked action, but levulose, maltose, and lactose were not far behind. The cells still remained spherical, however.

[11] Hamburger, H. J., Osmotischer Druck und Ionenlehre in ihrer Bedeutung für die Physiologie und die Pathologie des Blutes, Berlin, 1912.

[12] Weidenreich, F., *Folia Haematol.*, 1905, ii, 95.

[13] Hedin, Hamburger, Kozawa.

The best results were obtained with mixtures of Locke's solution and isotonic solutions of the sugars in water.[14] Isotonic mixtures are better than hypertonic, though slight variations in tonicity are well borne. The small amount of sugar present in Locke's solution, when it is made up after the usual formula, is far below that which exerts a preservative influence. In isotonic sugar solutions alone erythrocytes keep fairly well, but they sediment in a firm layer which can hardly be suspended without hemolysis. The sugar solution and Locke's solution to be mixed together must be autoclaved separately, because in mixtures of them the sugar caramelizes during the autoclaving.

Optimum Preservative Fluids.

For the cells of each species a special preservative mixture is required. In our experience the optimum preservative for the washed cells of the sheep is one made by adding to Locke's solution sufficient saccharose in isotonic, watery solution to give an ultimate concentration of 2.8 per cent of saccharose. Cells twice washed in gelatin-Locke's remain unhemolyzed in this sugar medium for at least 3 or 4 weeks; and even after 2 months hemolysis is exceedingly slight. As is well known, the cells begin to break down within 3 or 4 days when washed and kept in Locke's solution in the ordinary way. For their proper preservation, it is essential that they be washed in an abundance of fluid, since a trace of plasma soon leads to hemolysis. The properly washed and kept cells retain their color; they take up and give off oxygen readily; and though they sediment into a rather firm layer they are easily made into a uniform suspension which passes readily through a filter paper. The cells in such a suspension are discrete, not stuck together. They will withstand repeated washings, but after washing they break down somewhat sooner in Locke's solution than do fresh cells. Cells kept for 3 weeks and for a month have been used for the Wassermann reaction and compared with cells freshly obtained from the same sheep. They had the same hemolytic titer and gave identical results in the reaction.[15]

[14] Saccharose 10.3 per cent, dextrose 5.4 per cent.

[15] For these tests we are indebted to Dr. Russell L. Cecil.

Dextrose is a fairly good preservative for washed sheep cells and human cells. But the latter are best kept in a saccharose-Locke's mixture containing about 5 per cent of the sugar (4.9 per cent). We have thus preserved them for 4 weeks without any hemolysis in the supernatant fluid or on washing. They are easily suspended, and like sheep cells take up and give off oxygen readily. Cells of both sorts begin to break down in about 10 days in a plasma-Locke's-citrate mixture.

Washed rabbit cells remain unhemolyzed longest in plasma-Locke's-citrate, slightly over 2 weeks as a rule. In a mixture of Locke's with glucose and saccharose, containing 3 and 6 per cent of the sugars, respectively, they stay intact from 9 to 12 days.

Dog cells are most difficult to keep. They are very frail, and hemolyze rapidly in their own plasma. In a saccharose-dextrin-Locke's fluid containing 1.6 to 2.7 per cent of the sugar and 2 per cent of dextrin, they show no breaking down for from 5 to 12 days, when they have been thoroughly washed with gelatin-Locke's before being placed in this medium. Preservation for so long a time must be regarded as notable for elements which under ordinary circumstances, washed and kept in Locke's or sodium chloride solution, begin to hemolyze at once. 2 per cent of dextrin added to the Locke's-sugar mixture aids the preservation of dog cells; but it cannot be regarded as uninjurious, since in amounts of 10 per cent it causes a gradual browning of the blood pigment to methemoglobin. This alteration is not evident with 2 per cent dextrin.

We are inclined to attribute the hemolysis that eventually occurs in the optimum preservatives to autolysis within the substance of the cells. That it is not due to the action of the preservatives themselves is well shown by comparing the results with cells allowed to settle in these fluids and cells agitated each day. In such an experiment gelatin must be present to prevent mechanical injury. If the fluid used as a preservative is harmful the cells brought in daily contact with it by agitation break down much sooner than those left to sediment. This is what happens in Locke's solution, for example. But with fluids such as the saccharose-Locke's that is optimum for washed sheep cells, the sedimented and agitated cells show the same gradual breaking down.

Concomitant Factors.

There are many factors besides the character of the preservative which conceivably may affect the period of survival of the cells. Sedimented cells lose their oxygen within a few days; they are exposed to a possible digestive action of the leukocytic pellicle, and to possible injury of the living erythrocytes by contact with dead cells scattered in the mass. Cells that are stirred remain bright and are not exposed to the dangers mentioned. But in experiments specifically directed to these points, we have found that red cells deprived of oxygen last no longer than those well supplied with it, that the leukocytic pellicle of normal blood does not cause hemolysis of the erythrocytes, and that contact injury of living cells by the dead can be disregarded. *A priori* one might suppose that cells would keep best in a very small quantity of preservative fluid, since in this there would be less loss of their diffusible substances. But tests in which the cells were stirred daily and allowed to settle through long and short columns of fluid, have failed to show that this has any importance. It is our practice to place the washed cells in five or six times their bulk of preservative fluid and allow them to remain in sediment until wanted.

Preservatives Are Not Protectives.

If the preservative solutions protect the cells against mechanical injury, gelatin can be dispensed with and a single solution used throughout.

Experiment 7. Dextrose and Dextrin Are Not Protectives.—Dog blood was caught in Locke's-citrate as usual and the mixture distributed in equal amount in 5 centrifuge tubes. The cells of 4 were washed twice with ordinary Locke's solution, Locke's containing 5 per cent of dextrin (Merck), 3 per cent of dextrose, and 1½ per cent of gelatin, respectively, and were suspended to the original blood-citrate bulk in these fluids. With the 5th tube the form of washing was twice gone through, using the original fluid. All were now corked, shaken for 15 minutes, and centrifugalized. There was no hemolysis in the tubes containing gelatin-Locke's and plasma-Locke's-citrate, but in the others it was abundant and of about the same amount in all.

Plasma possesses both protective and preservative qualities, but it is, of course, no simple fluid.

The Preservation of Leukocytes.

Some tests were made to determine whether leukocytes require the same protective and preservative media as the red cells. An aleuronat exudate of the dog containing many large mononuclear cells was washed, half in gelatin-Locke's, half in ordinary Locke's solution, and portions were distributed in various fluids for preservation. As a control, the red cells of the same dog were similarly treated. After 1 week in the cold, all were washed, this time in ordinary salt solution, and the ability of the mononuclear cells to take up rat erythrocytes was tested, with fresh dog serum as complement. Only those cells which had been placed for keeping in the original citrated plasma now showed phagocytosis, and this was independent of whether gelatin had been present in the original wash fluid. Mononuclears kept in Locke's solution, and in the sugar-Locke's, and the sugar-Locke's-dextrin mixtures most favorable to the red cells, failed entirely to ingest the rat corpuscles.

The Preservation of Unwashed Red Cells.

The results with red cells gave some ground for the hope that the erythrocytes of blood received directly into a medium preservative for the washed cells and thus kept, would remain unhemolyzed longer than under the usual conditions. Experiments along this line were undertaken. It was necessary, of course, to use some anticoagulant, and for this purpose sodium citrate was employed. Considerable quantities of blood were taken, distributed with the preservative mixtures in large test-tubes, and kept in the cold. To estimate hemolysis not only was the color of the supernatant fluid noted but the cells were stirred up in $\frac{1}{8}$ per cent gelatin-Locke's and centrifugalized. This frequently brought to light a marked hemolysis of which there had been no trace when the cells lay in sediment. Our freezing point determinations showed that a watery solution of sodium citrate containing 3.8 per cent of the salt is isotonic with 0.95 per cent sodium chloride. Tests were first carried out for an optimum blood-citrate mixture.

Experiment 8. The Optimum Blood-Citrate Mixture for Rabbit Cells.—The blood of two normal rabbits, X and Y, was taken in portions of 3 cc. into sodium

TABLE III.

Hemolysis.

Mixture.	Rabbit X. 26 days.		Rabbit Y. 34 days.		Rabbit X. 34 days.		Rabbit Y. 47 days.	
1½ cc. blood +	Supernatant fluid.	Wash fluid.	Supernatant fluid.	Wash fluid.	Supernatant fluid.	Wash fluid.	Supernatant fluid.	Wash fluid.
1/20 cc. 10% sodium citrate.....	++	+++++	Clotted.		++		Clotted.	
2/16 " 10" " "	++−	++++	++−	++−	++−		++−	++++
2/16 "7.6" " "	++−	++++	+	++−	++−		+−	++
3/8 "7.6" " "	++−	+++	Tr.	++−	++−		Tr.	++
3/8 "3.8" " "	++−	++	+	Tr.	++−		+−	+
1 "3.8" " "	+	+	Ft. Tr.	"	+		0	Tr.
1½ "3.8" " "	Tr.	+−	0	Ftest. Tr.	+		0	"

citrate solutions of various concentration and amount. Each mixture was divided into two equal portions and tubed. After many days one tube of each sort was tested for hemolysis, and still later (after 47 days, all told, in the case of Y) the second was examined. The color of the supernatant fluid was recorded and also the amount of hemolysis when the cells were suspended in 6 cc. of $\frac{1}{8}$ per cent gelatin-Locke's and centrifugalized.

The results are shown in Table III.

As this experiment shows, the preservation of the erythrocytes of citrated rabbit blood is much influenced by the amount and concentration of the citrate. We have repeatedly found that the best results are obtained, not with the smallest amount of citrate that will prevent clotting, but with equal parts of blood and isotonic citrate solution. The same amount of a somewhat hypotonic solution (2.5 per cent citrate) also gives good results. With human blood, on the other hand, the proportion and concentration of the citrate seem to have little influence. With sheep and dog blood no quantitative tests have been made.

As Experiment 8 shows, when rabbit blood is mixed with the right amount of citrate its cells remain intact for a long time. The addition of sugars further increases the preservation and the real length of life of the cells to a slight extent, as our transfusion experiments have shown. Sheep cells keep no longer in the presence of sugar. But for human blood cells, it has a remarkable preservative effect.

Experiment 9. The Preservative Action of Sugars on the Cells of Citrated Human Blood.—20 cc. of two human bloods were taken, that from X in an equal bulk of $2\frac{1}{2}$ per cent citrate in water, that from Y in a citrate-salt solution containing 2 per cent of citrate, 0.3 per cent of sodium chloride, and the other salts of Locke's in the same relative proportion. To equal amounts of the blood-citrate mixtures, Locke's solution and isotonic saccharose and dextrose solutions, respectively, were added. One tube of each of these preparations was examined after 13 days and another after 20 days. The sedimented corpuscles were tested for concealed hemolysis by suspending them in $\frac{1}{8}$ per cent gelatin-Locke's and immediately centrifugalizing. The results are given in Table IV.

Experiment 10. The Preservative Action of Sugars on the Cells of Citrated Human Blood.—Three human bloods were taken in various amounts of citrate, as in the case of Experiment 8 with rabbit bloods. To one of the mixtures which contained 3 parts of blood and 2 parts of isotonic (3.8 per cent) citrate solution, 5 parts of isotonic dextrose solution were added. After 4 weeks all the preparations were examined as usual, the cells being suspended in gelatin-Locke's and centrifugal-

TABLE IV.

Hemolysis.

Mixture	Blood X. 13 days.		Blood Y. 13 days.		Blood X. 20 days.		Blood Y. 20 days.	
3 parts citrated blood +	Supernatant fluid.	Wash fluid.	Supernatant fluid.	Wash fluid.	Supernatant fluid.	Wash fluid.	Supernatant fluid.	Wash fluid.
0	+−	+−	Tr.	+−			Tr.	+
1 part Locke's.	Tr.	+−	+−	+−	Tr.	++−	+	+
3 " "	+−	+−	++−	++−	++−	++	++−	++
7 " "	++−	++	+++	+++	++	++++	++	+++
1 " saccharose.	0	Tr.	0	+−	Ft. Tr.	+−	Ftest. Tr.	+
3 " "	0	Ft. Tr.	0	+−	0	Tr.	0	+−
5 " " / 2 " Locke's.	Ft. Tr.	" "	0	+−	0	Tr.	0	+−
1 " dextrose.	0	" "	0	Ftest. Tr.	Ft. Tr.	0	Tr.	Tr.
3 " "	0	0	0	0	" "	0	Ft. Tr.	0
5 " " / 2 " Locke's.	0	0	0	0	" "	0	+	+−

ized. There was no hemolysis of those kept with dextrose, but those of the citrate mixtures all showed a marked breaking down, independent apparently of the amount of citrate present.

Other experiments confirm these results. It is safe to say that the red cells of normal human beings can be kept intact for nearly or quite 4 weeks, when 3 parts of the blood are taken directly in a mixture of 2 parts of isotonic sodium citrate and 5 parts of isotonic dextrose solution. With citrate alone, in any quantity, hemolysis is well marked in less than 2 weeks.

Locke's Solution Is Injurious.

The action of Locke's solution to cause hemolysis, which is so plain in the case of washed cells kept in it, is no less evident when this solution is added to citrated blood. Experiment 9 illustrates this fact for human blood.

Experiment 11. The Injurious Effect of Locke's Solution on the Cells of Citrated Rabbit Blood.—The blood of two rabbits, X and Y, was taken in an isotonic citrate solution, and to portions of the mixtures, Locke's solution and an isotonic watery solution of saccharose, respectively, were added. The tubes were examined for hemolysis after many days had elapsed (Table V).

TABLE V.

Hemolysis.

Mixture.	Rabbit X. 26 days.		Rabbit Y. 34 days.		Rabbit X. 34 days.		Rabbit Y. 47 days.	
3 cc. blood + 2 cc. citrate +	Supernatant fluid.	Wash fluid.	Supernatant fluid.	Wash fluid.	Supernatant fluid.	Wash fluid.	Supernatant fluid.	Wash fluid.
0	+	+	Ft. Tr.	Tr.	+		0	Tr.
3⅓ cc. Locke's.	++++	+−	+++	"	++++		+++	+
3⅓ cc. saccharose.	+	Tr.	0	0	+		Ft. Tr.	0

The greater the amount of Locke's solution mixed with the citrated blood the greater is the destruction of the corpuscles (Experiment 9). But as in the case of washed cells, the injurious action of the Locke's fluid can be completely prevented by means of a sugar.

Experiment 12. The Effect of Saccharose To Prevent the Injurious Action of Locke's Solution on Rabbit Corpuscles.—Rabbit blood was taken into isotonic citrate and to portions of the mixture Locke's solution, isotonic saccharose solution, and combinations of the two were added. After many days the tubes were examined for hemolysis in the usual way (Table VI).

TABLE VI.

Hemolysis.

Mixture.	Percentage in ultimate mixture.				Rabbit X. 26 days.		Rabbit Y. 34 days.	
3 cc. blood + 2 cc. citrate +	Blood.	Citrate.	Sugar.	Locke's.	Supernatant fluid.	Wash fluid.	Supernatant fluid.	Wash fluid.
0	60	40	−	−	+	+	Ft. Tr.	Tr.
3⅓ cc. saccharose.	36	24	40	−	+	Tr.	0	0
3⅓ cc. Locke's.	36	24	−	40	++++	+−	+++	Tr.
8½ cc. Locke's. 9½ cc. saccharose.	13	9	41	37	+	Ft. Tr.	0	0
17 cc. Locke's. 19 cc. saccharose.	7	5	46	42	Tr.	0	0	Tr.

A similar action of dextrose is indicated in Experiment 9 with citrated human blood.

SUMMARY.

The erythrocytes of some species are much damaged when handled in salt solutions, as in washing with the centrifuge after the ordinary method. The injury is mechanical in character. It may express itself in hemolysis only after the cells have been kept for some days. It is greatest in the case of dog corpuscles, and well marked with sheep and rabbit cells. The fragility of the red cells, as indicated by washing or shaking them in salt solution is different, not only for different species, but for different individuals. It varies independently of the resistance to hypotonic solutions.

The protection of fragile erythrocytes during washing is essential if they are to be preserved *in vitro* for any considerable time. The addition of a little gelatin ($\frac{1}{8}$ per cent) to the wash fluid suffices for this purpose, and by its use the period of survival in salt solutions of washed rabbit, sheep, and dog cells is greatly prolonged. Plasma, like gelatin, has marked protective properties.

Though gelatin acts as a protective for red cells it is not preservative of them in the real sense. Cells do not last longer when it is added to the fluids in which they are kept. Locke's solution, though better probably than Ringer's solution, or a sodium chloride solution, as a medium in which to keep red cells, is ultimately harmful. The addition of innocuous colloids does not improve it. But the sugars, especially dextrose and saccharose, have a remarkable power to prevent its injurious action, and they possess, in addition, preservative qualities. Cells washed in gelatin-Locke's and placed in a mixture of Locke's solution with an isotonic, watery solution of a sugar remain intact for a long time,—nearly 2 months in the case of sheep cells. The kept cells go easily into suspension free of clumps, they pass readily through paper filters, take up and give off oxygen, and when used for the Wassermann reaction behave exactly as do fresh cells of the same individual. The best preservative solutions are approximately isotonic with the blood serum. If the cells are to be much handled gelatin should be present, for the sugars do not protect against mechanical injury.

Different preservative mixtures are required for the cells of different species. Dog cells last longest in fluids containing dextrin as well as a sugar. The mixture best for red cells is not necessarily best for leukocytes.

A simple and practical method of keeping rabbit and human erythrocytes is in citrated whole blood to which sugar solution is added. In citrated blood, as such, human red cells tend to break down rather rapidly, no matter what the proportion of citrate. Hemolysis is well marked after little more than a week. But in a mixture of 3 parts of human blood, 2 parts of isotonic citrate solution (3.8 per cent sodium citrate in water), and 5 parts of isotonic dextrose solution (5.4 per cent dextrose in water), the cells remain intact for about 4 weeks. Rabbit red cells can be kept for more than 3 weeks in citrated blood; and the addition of sugar lengthens the preservation only a little. The results differ strikingly with the amount of citrate employed. Hemolysis occurs relatively early when the smallest quantity is used that will prevent clotting. The optimum mixture has 3 parts of rabbit blood to 2 of isotonic citrate solution.

In the second part of this paper experiments are detailed which prove that cells preserved by the methods here recorded function excellently when reintroduced into the body.

DISCUSSION.

Our findings show that in experiments with cells washed in salt solutions there is a large source of possible error in injury done during washing. That it has been so long overlooked by investigators is probably due to the fact that mechanical injury does not alter hemolytic titer, and furthermore that such injury often manifests itself only after the cells have been kept for several days. The reasons for the injury in salt solution and for the protective action of gelatin are not clear. We were led to employ the latter through some observations of Weidenreich,[16] who showed that red cells placed in salt solution containing gelatin do not become spherical as in ordinary salt solution but retain their normal shape. From this he concluded that the shape of the cell is determined not only by the osmotic pressure of the surrounding fluid, but by its molecular force (*Molecularkraft*), as determined through its content in colloidal substances. But the amount of gelatin which will confer protection in salt solution is far too little appreciably to alter the molecular force, or, as we have found, to preserve the normal shape of the cell. For this latter effect, at least

[16] Weidenreich, F., *Folia Haematol.*, 1905, ii, 95.

as much as the $1\frac{1}{2}$ per cent of gelatin, recommended by Weidenreich, is necessary. In Locke's solution containing $\frac{1}{8}$ per cent of gelatin the cells are spherical. In the preservative sugar-Locke's solutions also the cells are spherical. It is interesting that cells kept for many days in such distorted condition should retain their usefulness for the body.

The ability of plasma to protect red cells against mechanical injury may explain to some extent the relatively long survival of the latter in the circulation. A clinical investigation of the mechanical fragility of red cells as determined by shaking experiments might yield results of value.

The preservative action of the sugars on red cells kept *in vitro* is largely dependent, when Locke's solution is present, on a prevention of injury from this latter. That Locke's solution should fail to be a physiological medium for red cells is scarcely surprising. Much recent work has gone to show that for the cells of different organs, different solutions are physiological. But Locke's solution is not merely lacking in some constituent needed by the red cells. It is actively injurious. This is well seen in the experiments with citrated blood, as also in the action of sugars to prevent the injury. The further preservative action of the sugars is perhaps referable to their ability to retard proteolytic digestion;[17] and the peculiarity that it is effective only in the case of certain red cells, to the fact that the erythrocytes of different species have a somewhat different permeability for sugars.[18]

Sheep cells washed and kept according to our methods are as suitable for the Wassermann reaction as fresh cells. But considerable manipulation of them is required. Formalization of the defibrinated blood, as practiced by Armand-Delille and Launoy,[19] and by Bernstein and Kaliski,[20] would seem more practical. The blood-citrate-saccharose mixture should prove useful for the preservation of rabbit and human corpuscles for culture media.

[17] Ogáta, M., *Arch. f. Hyg.*, 1885, iii, 204.

[18] Kozawa, S., *Biochem. Ztschr.*, 1914, lx, 146, 231.

[19] Armand-Delille, P., and Launoy, L., *Ann. de l'Inst. Pasteur*, 1911, xxv, 222.

[20] Bernstein, E. P., and Kaliski, D. J., *Ztschr. f. Immunitätsforsch., Orig.*, 1912, xiii, 490.

THE PRESERVATION OF LIVING RED BLOOD CELLS IN VITRO.

II. The Transfusion of Kept Cells.

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In the foregoing paper methods are described whereby red blood cells may be kept intact for long periods *in vitro*. It has remained to determine whether cells kept according to these methods are alive in the sense that they are capable of functioning in the animal body. This can be told by transfusion of the kept cells in bulk, with appropriate control. We have performed many such experiments, using rabbits.

The cells for preservation were obtained by bleeding etherized rabbits from the carotid or aorta into a Locke's-citrate mixture or an isotonic solution of citrate (3.8 per cent) in water. In some instances, the cells were washed, with gelatin-Locke's ($\frac{1}{8}$ per cent gelatin), and placed in a preservative solution; in others they were kept, without washing, in the original mixture of citrate and blood, or in citrate and blood plus a sugar. When they were to be used the supernatant fluid was pipetted away; and sometimes the sediment was suspended directly in Locke's solution to the original blood bulk and used for injection. More often it was washed once or twice with gelatin-Locke's and then suspended in ordinary Locke's. The suspension was filtered through two thicknesses of gauze; warmed to body temperature; and introduced under slight pressure into the ear vein of a rabbit which had just been bled.

The rabbits which furnished and received the cells were selected from a number tested against one another to rule out the presence of iso-agglutinins and isolysins. In the rabbit such antibodies are weak and infrequent. None of our results suggest their action. The animals chosen as recipients were bled in large amount from the ear

and the hemoglobin loss was replaced by the transfusion of kept cells. The bleeding and injection were repeated if the amount of kept cells allowed, as in instances when the cells from two rabbits had been preserved for the injection of a single individual. In every case a large proportion of the blood was drawn and replaced. The fate of the transfused cells was followed by blood counts, hemoglobin estimations, direct microscopic observations on the appearance of the blood elements, and by daily tests of the urine for hemoglobin (guaiac and spectroscopic tests) and bile (Gmelin and Bouvais tests). In addition, the percentage of reticulated red cells in circulation was noted in wet preparations stained with cresyl blue. This was done because an increase in the reticulated cells indicates an abnormal activity on the part of the bone marrow, such as would be the result of an increased destruction of the circulating erythrocytes. The rabbit's temperature was taken twice daily and its weight frequently recorded.

Some of the results may be briefly summarized and thus a repetition of data avoided. The bleeding and replacement of blood were always well borne. They took from one-half to three-quarters of an hour, and at the conclusion the animal usually went at once to eating. There was never a suppression of urine. Bile was never found in it and blood only in one instance, in a control experiment. The rabbit was, in this case, injected with cells kept, not in a preservative, but in Locke's solution, and a copious breaking down resulted with hemoglobinemia, hemoglobinuria, and death (Experiment 6).

In the specimen protocols that follow, the amount of blood drawn and replaced is recorded in percentage of the animal's total hemoglobin content. This is not to be confused with the percentage of hemoglobin in the circulating blood as obtained with the hemoglobinometer. It gives a much more precise idea of the proportion of formed elements dealt with, than would the blood volume. As is well known, when bleeding is done, even very rapidly, the last portion of blood contains much fewer cells than the first.[1] In thirteen instances in which we have taken into citrate from 30 to 50 per cent of the total calculated volume of a rabbit's blood and have com-

[1] Boycott, A. E., in Pembrey, M. S., and Ritchie, J., Text-Book of General Pathology, New York and London, 1913, 9.

pared the hemoglobin concentration of the drawn blood with that of the animal originally, it was found to be on the average 17 points lower by the Sahli scale. In general we have noted both the volume and the hemoglobin of the drawn blood, and have calculated from these the amount of cell suspension of known hemoglobin content that should be injected to restore the animal's hemoglobin content to the normal. In some of the early experiments this was not done and replacement was made by volume. According to Boycott and Douglas[2] a rabbit possesses 5.5 cc. of blood for 100 gm. of body weight. We have used this figure with hemoglobin estimations by the Sahli scale in determining the proportion of total blood pigment, and thus of erythrocytes, drawn and replaced. Since only 93 per cent of a rabbit's total hemoglobin is in the circulating blood (Boycott

TABLE I.

Time before or after transfusion.	Red blood corpuscles.	Hemoglobin.
		per cent
Before	4,890,000	87
3 hrs. after	4,590,000	85
24 " "	4,460,000	85
3 days "	4,510,000	83
6 " "	4,450,000	81

and Douglas), our estimate must be considered as rather less than the actual proportion. When two withdrawals and injections were carried out, allowance was made in the calculations for the mixed character of the second portion of blood withdrawn.

Experiment 1. Transfusion with Washed Cells Kept in a Dextrose-Locke's Solution and Washed before Use.—An animal weighing 1,700 gm. was bled 41 cc., and 41 cc. of a suspension of kept blood cells at once injected. By calculation some 30 per cent of the total hemoglobin was thus taken and replaced. The kept blood had been taken eight days previously, twice washed, and placed in Locke's solution to which 2½ per cent of glucose and ½ per cent of gelatin had been added. It was washed twice just previous to injection and suspended in Locke's solution. This handling brought about no hemolysis (Table I).

[2] Boycott, A. E., and Douglas, C. G., *Jour. Path. and Bacteriol.*, 1909, xiii, 256.

The animal was killed on the sixth day because of an infected ear. The reticulated red cells were not followed in this case. Fresh preparations of the blood failed to show shadows.

Experiment 2. Transfusion with Cells Kept in a Blood-Citrate-Saccharose Mixture and Washed before Use.—For this experiment the erythrocytes of two rabbits were used after they had been kept separately in mixtures of the citrated blood with saccharose for 11 and 12 days, respectively. Three parts of the blood were mixed for keeping with 2 of isotonic citrate and 5 of isotonic saccharose solution. The cells were washed once just before injection and suspended in Locke's solution. Following the injection there was no hemoglobin in the supernatant or wash fluid. The rabbit that acted as recipient was twice bled (40 and 6 cc.) and twice injected. 31 per cent of the animal's hemoglobin was thus replaced (Table II).

TABLE II.

Time before or after transfusion.	Red blood corpuscles.	Hemoglobin.	Reticulated cells.	Weight.	Urine.	Temperature.
		per cent		*gm.*	*cc.*	°*F.*
1 day before	6,500,000	91	25 in 500	2,025	65	102
Just before	6,100,000	90	19 " "		38?	102
3 hrs. after	6,500,000	90				
1 day "	6,640,000	87	24 " "		150	102.6
2 days "	6,540,000	86	14 " "		80	101.9
3 " "	6,540,000	83	12 " "	2,050	150	101.9
4 " "	6,840,000	91	13 " "		180	101.6
5 " "	6,420,000	85	11 " "		353	101.9
7 " "	6,730,000	89	9 " "		110	101.8
9 " "	6,640,000	90	11 " "	1,875	80	101.9

Fresh preparations of the blood made daily appeared normal throughout. On the third day after the transfusion a slight polychromatophilia was noted.

Experiment 3. Transfusion with Cells Kept in a Blood-Citrate Mixture and Washed before Use.—The blood of two rabbits was taken into citrate as usual (3 parts of blood to 2 parts of citrate) and kept for 11 days. The cells were washed once just prior to injection and suspended in Locke's solution. There was a slight hemolysis in the supernatant and wash fluids. The amount was calculated by comparing the color of these fluids with a laked preparation of the ultimate suspension, and it was found to equal about 0.2 per cent of this suspension. The recipient rabbit was bled 60 cc., or 36 per cent of the total hemoglobin content, and an equivalent amount was injected (Table III).

Throughout in fresh preparations the blood appeared normal. On the second day after transfusion occasional polychromatophilia was noted.

TABLE III.

Time before or after transfusion.	Red blood corpuscles.	Hemoglobin.	Reticulated cells.	Weight.	Urine.	Temperature.
		per cent		*gm.*	*cc.*	*°F.*
3 days before	6,320,000	87	12 in 500	2,250		101.8
1 day "	6,480,000	85	14 " "		70	101.8
Just "	6,010,000	80	18 " "		35	102.0
5 hrs. after	6,460,000	85				
1 day "	6,380,000	86	9 " "		60	101.4
2 days "	6,820,000	90	16 " "		35	102.2
3 " "	6,820,000	86	11 " "		64	102.2
4 " "	6,440,000	86	6 " "		51	101.9
5 " "	6,560,000	87	8 " "	2,375	140	101.5
7 " "	6,850,000	86	10 " "		250	101.2
9 " "	7,390,000	93	5 " "		172	101.4
11 " "	6,720,000	88	6 " "	2,300	170	102.0

Experiment 4. Transfusion with Cells Kept in a Blood-Citrate-Saccharose Mixture and Suspended Directly in Locke's Solution.—The blood of two rabbits was taken and kept as in Experiment 14 for 13 and 15 days, respectively. The supernatant fluid was pipetted off just prior to injection and the cells suspended in Locke's solution without washing. Two bleedings (of 40 and 50 cc.) were done and two injections. About 64 per cent of the total hemoglobin was thus withdrawn and more than replaced, as the blood examination showed (Table IV).

On the 2nd day after operation there were slight anisocytosis and polychromatophilia.

TABLE IV.

Time before or after transfusion.	Red blood corpuscles.	Hemoglobin.	Reticulated cells.	Weight.	Urine.	Temperature.
		per cent		*gm.*	*cc.*	*°F.*
1 day before	4,860,000	68	16 in 500	1,800		103.4
Just "	4,790,000	65	20 " "		150	103.2
3 hrs. after	5,670,000	81				
1 day "	5,220,000	76	16 " "		130	102.6
2 days "	5,280,000	73	21 " "		10?	103.0
4 " "	5,360,000	75	19 " "	1,825	170	102.4

Many experiments similar to these were done and with the same general results. Rabbit red blood cells kept for two weeks *in vitro* under suitable conditions can be used with good results to replace the blood lost in a hemorrhage. It is unnecessary to wash the cells which

may be simply suspended in Locke's solution after the preservative mixture is pipetted off. The preservative mixture which we have found best,—blood plus sodium citrate plus an isotonic saccharose solution—cannot be injected with the cells because of its content in citrate, but the small portion of it that remains with the cells after pipetting is not harmful. In blood-citrate mixtures without sugar the cells show some slight hemolysis (Experiment 2) after 2 weeks; and in several instances a slight drop in the cell count and hemoglobin percentage following transfusion, together with a rise in the number of reticulated red cells, has indicated that the kept cells were disappearing from the circulation and that the bone marrow was active in repairing the loss.

TABLE V.

	Time.	Red blood corpuscles.	Hemoglobin.	Reticulated cells.
			per cent	
Rabbit A.				
35 cc. taken of the total 78 cc. of blood.	Before operation.	5,500,000	106	2 in 500
	Day after "	2,400,000	48	53 " "
	19 days after "	5,000,000	77	27 " "
Rabbit B.				
45 cc. taken of the total 139 cc. of blood.	Before operation.	6,100,000	112	1 " "
	Day after "	3,600,000	65	15 " "
	18 days after "	5,870,000	110	14 " "
Rabbit C.				
42 cc. taken of the total 94 cc. of blood.	Before operation.	6,800,000	125	2 " "
	Day after "	3,000,000	54	13 " "
	25 days after "	6,000,000	112	35 " "

In striking contrast to these results are some that were obtained in control experiments.

Experiment 5. Effects of Bleeding Alone.—Two rabbits were bled as usual, but received no injection afterwards. Both died within a few minutes. The calculated blood volume of the animals was 88 and 60 cc., and the bleedings were for 50 and 28 cc., respectively.

Experiment 6.. Effects of Bleeding Followed by Injection of Locke's Solution.—Three rabbits were bled and an equivalent amount of Locke's solution was injected intravenously. There was an immediate great drop in hemoglobin percentage and number of red cells. Regeneration was still incomplete after many days (Table V).

Experiment 7. Bleeding Followed by Transfusion of Cells Washed and Kept in Locke's Solution.—A rabbit with a calculated blood volume of 87 cc. was twice bled (44 and 28 cc.) and an equivalent amount of kept cells was introduced. Thus about 56 per cent of the total hemoglobin was replaced. The kept cells had been washed and preserved in Locke's solution for 11 days and they were again washed just previous to injection. At this time some hemolysis was noted. The animal died in less than 24 hours after the injection and on autopsy there were hemoglobinuria, hemoglobinemia, spodogenous spleen, and other findings typical of the breaking down of blood in large quantities.

These control rabbits all fared badly. Evidently the aid rendered by a transfusion of cells kept in a proper preservative is a real one. As Experiments 4 and 5 show, these cells function normally even after they have been kept *in vitro* for 2 weeks. We have performed a number of transfusions with cells kept longer. They remain unhemolyzed for as long as 4 weeks, but by the end of the 3rd week have largely lost their ability to be useful when reintroduced into the body, as shown by the fact that within a few days they disappear from the circulation. This disappearance is unaccompanied by any signs of hemolysis or, indeed, of other derangement. The animal eats well and may gain weight. The anatomical findings in such cases have interest as bearing on methods of blood destruction. Discussion of them will be reserved for another paper. From among the many experiments one will be given here to illustrate the facility with which the body disposes of blood elements no longer useful in the circulation.

Experiment 8. Transfusion with Cells Preserved Too Long in Vitro.—A rabbit weighing 1,925 gm. was bled 54 cc. and transfused with kept cells. 44 per cent (actual) of the total hemoglobin was thus taken and an amount equal to only about 37½ per cent put back. By calculation this should have caused a fall in hemoglobin percentage as determined with the Sahli instrument to 75 per cent after the transfusion, and that indeed was the figure obtained. The cells had been kept for 23 days in a mixture of 3 parts of blood, 2 parts of isotonic citrate solution, and 5 parts of isotonic saccharose solution. For injection they were washed once in gelatin-Locke's solution and suspended in ordinary Locke's. Neither the supernatant nor the wash fluids showed the slightest trace of hemolysis (Table VI).

There was at no time bile or blood in the urine. On the 2nd and 3rd days after the transfusion, there was marked polychromatophilia and some anisocytosis. Otherwise the animal seemed normal. The blood examinations on the 2nd day indicated that nearly all the transfused cells had disappeared from the

TABLE VI.

Time before or after transfusion.	Red blood corpuscles.	Hemoglobin.	Reticulated cells.	Weight.	Urine.	Temperature.
		per cent		*gm.*	*cc.*	*°F.*
4 days before	5,960,000	78	9 in 500			101.6
3 " "	5,850,000	81	12 " "	1,925	20?	101.8
1 day "					280	
Just "	5,810,000	80	8 " "			102.7
3 hrs. after	5,490,000	75				
1 day "	4,380,000	58	52 " "		100	102.2
2 days "	3,890,000	51	45 " "		120	101.8
3 " "	3,900,000	59	44 " "	1,790	20	101.4

circulation. The rabbit was killed on the 3rd day. The organs appeared normal. The spleen weighed only 0.7 gm. It gave a well marked iron reaction, and showed many phagocyted red cells.

A transfusion such as the above with intact cells kept too long *in vitro* is not helpful, or only indirectly so as supplying the constituents for new red cells; but in our experience it is not harmful.

The Preservation of Human Red Cells.

Human red cells can be preserved *in vitro* much longer than rabbit cells; and there seems little doubt that they could be profitably used for transfusion. Recently Weil[3] has reported upon a number of transfusions with whole citrated human bloods kept in the cold for several days. He employed 1 part of 10 per cent citrate to 10 parts of blood. But Lewisohn[4] has shown that citrate in this amount is dangerous to the organism; and we have found that human blood thus kept with citrate begins to hemolyze in about a week, the hemolysis being more dangerous because it is often completely concealed amid the sedimented corpuscles. Cells kept in a blood-citrate-dextrose mixture, according to the method detailed in the first part of this work,[5] remain

[3] Weil, R., *Jour. Am. Med. Assn.*, 1915, lxiv, 425.

[4] Lewisohn, R., *Surg., Gynec. and Obst.*, 1915, xxi, 37.

[5] Three parts of blood are caught in a mixture of 2 parts of isotonic sodium citrate solution (3.8 per cent) in water, and 5 parts of isotonic dextrose solution (5.4 per cent) in water. The preparation is allowed to remain undisturbed in the ice box until wanted, when the supernatant fluid is drawn off and the sedi-

intact for 4 weeks; and it would seem preferable to use cells thus kept, suspending them for injection in a little salt solution. By this method the plasma is lost; and it is of great importance for some conditions in which transfusion is employed. But in cases of simple exsanguination, as Abel and his coworkers have shown,[6] to furnish corpuscles to the body is sufficient. One might ask whether the slight remnant of plasma, sugar, and citrate remaining with the corpuscles and injected with them would be harmful. Our experiments with rabbits, and recent work on the injection of citrate and concentrated sugar solutions[7] prove this danger to be negligible. The experiments with rabbits show that if blood cells are kept too long to function, but are still intact when restored to the circulation, they are easily disposed of by the body. Tests for iso-agglutinins and hemolysins would be necessary before the transfusion of kept human cells.

SUMMARY.

In order to determine the availability for functional uses of red cells kept *in vitro* by our methods, transfusion experiments have been carried out with rabbits by which a large part of their blood was replaced with kept rabbit cells suspended in Locke's solution. It has been found that erythrocytes preserved in mixtures of blood, sodium citrate, saccharose, and water for 14 days, and used to replace normal blood, will remain in circulation and function so well that the animal shows no disturbance, and the blood count, hemoglobin, and percentage of reticulated red cells remain unvaried. Cells kept for longer periods, though intact and apparently unchanged when transfused, soon leave the circulation. Animals in which this disappear-

ment of cells suspended in Locke's solution. The slight whitish flocculus which is sometimes present above the sediment disappears in the Locke's solution. It has been our practice to filter the suspensions of kept rabbit cells through gauze, previous to use. Needless to say, the preparation must be kept sterile. The sugar and citrate solutions should be autoclaved separately, or the mixture of them can be put through a Berkefeld filter.

[6] Abel, J. J., Rowntree, L. G., and Turner, B. B., *Jour. Pharmacol. and Exper. Therap.*, 1914, v, 625.

[7] Hustin, A., *Ann. et bull. Soc. roy. d. sc. méd. de Bruxelles*, 1914, lxxii, 104. Enriquez, *Presse méd.*, 1914, xxii, 121.

ance of cells is taking place on a large scale, remain healthy save for the progressing anemia. The experiments prove that, in the exsanguinated rabbit at least, transfusions of cells kept for a long time *in vitro* may be used to replace the blood lost, and that when the cells have been kept too long but are still intact they are disposed of without harm. The indications are that kept human cells could be profitably employed in the same way.

THE CAUSE OF RAT-BITE FEVER.

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PLATE 33.

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Four cases of rat-bite fever have recently come under our observation, and we have been able to make a systematic study of two of them.

The symptoms of the disease, which usually begin after an incubation period of from 10 to 27 days, consist of chills, fever, headache, and malaise. Inflammation is soon observed at the site of the apparently healed bites, with pains in the limbs on the affected side of the body, dark red skin eruptions, and swelling of the lymph glands. The attack with its local manifestations and high fever continues for from 3 to 7 days, the duration varying in individual cases. It alternates with an afebrile interval of 2 or 3 days. All the cases that came under our observation showed this picture, which may be considered typical of rat-bite fever.

Our investigations were conducted in the following manner. On August 9, 1915, when one of the patients was showing active symptoms, a sterile excision was made of a small piece of skin from the arm. This piece of skin showed the typical exanthem described above. The excised tissue was placed under dark-field illumination and examined microscopically, and in it were observed numerous actively moving spirochætæ. The skin tissue and also blood drawn from the patient were inoculated into monkeys, guinea pigs, and white rats. It was possible to infect all the animals inoculated in this manner, and to transmit the disease from them to other animals.

The study with the second patient was even more detailed. During the second attack a swollen lymph gland was punctured, and an India ink preparation made of the exudate, according to the

method of Burri. At the same time, a section of the excised lymph gland was impregnated with silver, according to Levaditi's method. The spirochætæ were identified in both preparations (Fig. 1).

The organism is somewhat larger than *Spirochæta pallida*, but smaller than *Spirochætæ duttoni* and *obermeieri*.

One of the patients was treated with mercury, and the other with salvarsan. Both recovered. In treating these cases we recalled the fact that in recurrent fever, one of the spirochætal diseases which shows a temperature curve similar to that of rat-bite fever, salvarsan has proved effective. Syphilis, also, which resembles rat-bite fever in swelling of the lymph glands and skin eruptions, is improved or cured by salvarsan or mercury.

Hence, not only on the basis of the microscopic findings, but also by comparison with related diseases, we are led to the conclusion that the spirochæta discovered by us is in all probability the cause of rat-bite fever. Further investigations on the subject are in progress, and we reserve the privilege of making more definite statements in the future.

EXPLANATION OF PLATE 33.

FIG. 1. Section of a lymph gland impregnated with silver nitrate according to Levaditi's method. In the center is seen a mass of spirochætæ.

FIG. 1.

A CONTRIBUTION TO THE BIOLOGY OF PERIPHERAL NERVES IN TRANSPLANTATION.

II. Life of Peripheral Nerves of Mammals in Plasma.

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Plates 34 to 41.

(Received for publication, July 21, 1915.)

Nageotte[1] has demonstrated that in pieces of peripheral nerves removed from the living animal and kept *in vitro* under certain conditions, the first stages of Wallerian degeneration may occur exactly as in the peripheral part of a divided nerve in the living organism. The phenomena are identical, but the process takes place more quickly *in vitro*. The conditions required for this degeneration *in vitro* are the presence of a solution of sodium chloride with some salt of bivalent metals (calcium, strontium, or magnesium) in suitable proportions, such as Ringer solution.

Nageotte found that the incubation of nerve fibers in isotonic solution of sodium chloride for twenty-four hours left them unaltered, and if they were put in Locke's solution after several days they degenerated exactly as if they were put directly into this fluid. I have observed a typical Wallerian degeneration in nerve fibers incubated in homologous and heterologous serum at body temperature for twenty-four hours, and in this case, as well as in Locke's solution, the degeneration takes place much more quickly than in the organism. One would expect, therefore, a Wallerian degeneration as a result of the incubation of peripheral nerves in plasma, but this did not take place.

[1] Nageotte, J., *Compt. rend. Acad. d. sc.*, 1910, cl, 731; *Compt. rend. Soc. de biol.*, 1910, lxix, 556.

EXPERIMENTAL.

The experiments were performed in the following way. From a living rabbit pieces $\frac{1}{2}$ to $\frac{2}{3}$ cm. long were removed from the sciatic nerve, and the nerve fibers were dissociated in Ringer solution by means of needles. The fibers were put immediately into a drop of plasma on a cover-glass, which was inverted over a hollow slide, sealed with paraffin, and incubated at 38° C. The preparations were observed from day to day under the microscope on a heated table and no change in the fibers was noticed up to the 10th or 12th day after incubation (Figs. 1 and 2). In hardened and stained preparations the nerve fibers had the same appearance on the 6th, 7th, and 8th days as on the 1st day.

I attempted to produce a degeneration in the nerves by adding calcium chloride to the plasma, but the results were the same. One drop of a 1 per cent solution of calcium chloride was added to 5, 7, and 8 drops of plasma, and from each of these mixtures a series of preparations were incubated at 38° C. As a control 1 drop of 1 per cent solution of calcium chloride was added to 5, 7, and 8 drops of serum and to 5, 7, and 8 drops of Ringer solution in which nerve fibers were incubated.

In the nerve fibers from the calcium plasma no degeneration occurred, whereas the nerves from the calcium serum and calcium Ringer solution tubes developed a degeneration in the usual way.

It is well known that pieces of connective tissue, glandular organs, skin, etc., when incubated for some time in plasma give rise to a growth of tissue around the original fragment, in some cases of the same specific cells as those constituting the original piece, but more often built of cells resembling the connective tissue cell type. Accordingly, it was to be expected that in the experiments a growth of tissue would occur originating from the cells of Schwann, but this did not take place. I prepared 80 cultures of nerve fibers removed from the sciatic nerve of living rabbits and in none was growth observed up to the 16th day of incubation.

I then cut the sciatic nerve of a rabbit, closed the wound, and twenty-four hours later removed a piece from the peripheral part. From this piece of nerve which had developed the first faint changes

of the Wallerian degeneration 10 cultures were prepared. No growth was observed from these nerve fibers.

Another series of 32 cultures was prepared, in the usual way with a hanging drop of plasma in hollow slides, from the peripheral part of a divided nerve 2 days after section. There were no changes in the fibers, and no growth was observed up to the 16th day.

Twelve cultures were prepared from the peripheral part of a divided nerve 4 days after section, and there was no growth.

In a series of 36 cultures prepared from the peripheral part of a divided nerve 5 days after section different results were recorded. In 6 of these 36 cultures I observed a growth of characteristic appearance.

After incubating the cultures for 3 and 4 days a number of thin filaments consisting of light-breaking, slightly granular protoplasm were seen growing out from the cut ends of the nerve fibers. In the beginning they have a conic or tapering shape ending in a point in which are seen ameboid movements, by means of which they emerge into the plasma, gain in length, and soon take on a more or less regularly cylindrical appearance measuring in width the same as or slightly less than the individual nerve fibers from which they have grown out. They branch freely, and from the 5th day they assume other characteristics, which are, however, best studied after fixation and staining.

Some of the cultures were hardened in formalin (2 per cent) and subsequently stained in hematoxylin. Others were hardened by means of the Held pyridin method and stained by Cajal's silver nitrate method.

Preparations Stained by Hematoxylin.—(Figs. 3, 4, and 5.) The original piece of nerve is 3 to 8 mm. long and about $\frac{1}{2}$ to 1 mm. wide. In its two ends the individual nerve fibers are easily discernible and display the ordinary appearance of Wallerian degeneration from this stage (that is, on the 5th day). Included in the sheaths of Schwann the following structures are seen: myelin ovoids containing fragments of the axis cylinder, and between 2 ovoids the nuclei of Schwann dislodged into the center of the fiber and embedded in protoplasm. From the cut ends of the individual nerve fibers the newly grown structures enter the plasma. In many of the fibers that give rise to growth, the cross-section of the fiber made by its removal from the

animal has passed close by a nucleus of Schwann, and such a cell marks the end of the old fiber and the beginning of the young one. In only a few of the old fibers a nucleus of Schwann is not present at the end, but in these cases it is found in the fiber only a few microns further in toward the center.

When they reach the plasma, the newly grown protoplasmic structures which form a direct outgrowth from the perinuclear protoplasm (syncytium) of Schwann of the old fibers are slightly fibrillary or granular. They have a tapering shape and a width averaging at their starting point 6 to 8 microns; that is, slightly less than the fibers from which they have grown out. Their tree-like branches are much thinner than the main stem and end in a point or a little bulb, round or oval in shape. In some of the newly grown filaments from distance to distance a well outlined and darkly stained nucleus is seen. These nuclei are oval or fusiform with their longest diameter in the direction of the filament; their shortest (transverse) diameter is ordinarily larger than the filament itself and the presence of the nuclei in the filaments produces varicosities of a corresponding size.

In some of the filaments 3 to 4 nuclei appear in a row one after another embedded in a granular column of protoplasm without any membrane, the whole picture resembling the contents of the sheaths of Schwann in a degenerating nerve 14 to 15 days after section. To complete this likeness we find included in some of the grown out threads small agglomerations of fatty (myelin) globules of different volume from that of minute granules up to or even surpassing the size of a nucleus of Schwann, embedded in varicose accumulations of protoplasm and generally beside a nucleus of Schwann.

In some preparations the filaments after reaching the plasma anastomose between each other, and thus form a real framework-like syncytium (Fig. 4). After 6 days of incubation the newly grown filaments are about 500 microns in length.

Silver-Impregnated Preparations.—The Held pyridin method was found to be the only practicable one for fixing the cultures. It causes no precipitation in the plasma and the subsequent staining by Cajal's silver nitrate method has given good results.

The original fragments of nerves stain dark brown and the surrounding plasma a light yellowish brown. The filaments which have

grown out are slightly darker brown than the plasma and are easily discernible against this background. In no case did the newly grown filaments assume the black, or dark brown color which is characteristic of axis cylinders treated according to this method, which I have demonstrated growing out from pieces of cerebellum and spinal ganglia incubated in plasma.

Morphologically the preparations display the same appearance as the hematoxylin preparations.

The newly grown filaments emerge from the transversely cut ends of the nerve fibers, at the end of which a nucleus of Schwann surrounded by protoplasm is often observed; or such a cell is seen a little further in toward the center of the piece, or in the young fiber a few microns from its base. The filaments are of different sizes, measuring in width from 2 to 3 microns up to 8 to 9 microns at the point where they enter the plasma. From this point they steadily decrease in size and in their course through the plasma they curve and bend in various directions, although as a rule the main direction in the beginning of their growth at least is parallel to the nerve fibers of the original fragment. The filaments are for the most part unevenly shaped and provided with large and small varicosities. The large varicosities include one and sometimes two oval nuclei of the Schwann nucleus type, the small varicosities are formed by accumulation of granular protoplasm. Some of the largest filaments divide into two or three branches which part from the main stem at acute angles, usually leaving a little varicosity of protoplasm at the point of division. The finest branches are provided with minute varicosities and they end in a point or in a little granular protoplasmic bulb.

In a series of 20 cultures prepared from the peripheral part of a divided nerve 6 days after section a growth of filaments was observed in 5. The newly grown structures had the appearance described above (Figs. 6 and 7).

I prepared cultures from the peripheral part of a divided nerve, 8, 10, 13, 15, and 19 days after section. The results of these experiments are shown in Table I.

The following conclusions may be drawn from these experiments. Plasma cultures prepared from normal peripheral nerves give rise

to no proliferation of cells. Plasma cultures prepared from the peripheral part of a divided nerve within the first 4 days after section give rise to no growth. 16 per cent of plasma cultures prepared from the peripheral part of a divided nerve 5 days after section give rise to a proliferation in the plasma of the syncytium of Schwann, and in series of cultures prepared from such a divided nerve in Wallerian degeneration there is a direct ratio between the stage of the Wallerian degeneration counted in days and the percentage of positive cultures as far as the growth of syncytium of Schwann is concerned.

The growth in plasma of the syncytium of Schwann from nerve fibers in a Wallerian degeneration of a more progressed stage, from the

TABLE I.

Cultures of the Peripheral Part of a Divided Nerve in Different Stages of Wallerian Degeneration.

Stage of Wallerian degeneration (No. of days elapsed after section).	Total No. of cultures.	No. of cultures in which growth had occurred.	Percentage of growth.
days			
1, 2, and 4.....	54	None	0
5.....	36	6	16.6
6.....	20	5	25.0
8.....	20	6	30.0
10.....	20	8	40.0
13.....	24	11	45.8
15.....	21	16	76.0
19.....	17	14	82.3

13th day for instance, displays minor variations from what has been described concerning the growth from fibers in an earlier stage. The growth is more abundant and the rate of growth is quicker. The fibers are mostly parallel; they are much thinner in their whole length and are far more richly provided with nuclei of the ordinary oval Schwann nucleus type. Around many of these nuclei there is an agglomeration of fatty (myelin) granules embedded in scant protoplasm. The filaments from the later stages of Wallerian degeneration are not so liable to branch, and anastomoses are not seen (Figs. 8, 9, 10, and 11). In Cajal preparations from this stage too, the fibers stain light brown and none of them take the black or dark brown

color which is characteristic of the axis cylinders. Besides the filaments a migration and growth of ordinary connective tissue cells (Fig. 3) are found in many of the cultures.

I should mention here the results of my experiments on the cultivation of pieces of the central nervous system and spinal ganglia of young mammals. In 1913[2] I observed that the brains of chick embryos, of cats 6 weeks old, of rabbits 2 months old, and of dogs 3 weeks old, when cultivated in plasma developed long (1.25 mm.) protoplasmic filaments, which were studied mainly from living preparations and were interpreted as nerve fibers. Such structures developed also from spinal ganglia of rabbits 7 months old and from the spinal cord of cats 6 weeks old. In a later communication,[3] after having succeeded in staining the preparations by means of Cajal silver impregnation, I stated in confirmation of my preliminary report, that nerve fibers grow out from pieces of cerebellum and spinal ganglia of young cats and guinea pigs when cultivated in plasma, and that these nerve fibers did not anastomose, and extended into the plasma unaccompanied by structures of any kind.

Certain other phenomena of growth were also observed by means of the silver impregnation which made possible a discrimination between two different kind of filaments, one of which was nerve fibers and the other probably neuroglia. The proof that nerve fibers grew into the plasma was based upon the direct and continual extension of the black silver-stained nerve fibers of the original pieces into the plasma, where they formed evenly cylindrical or uneven and varicose, mostly branching filaments, which were never granular and did not anastomose.

Besides these fibers an abundant growth of syncytial protoplasm was observed. In unstained living preparations it is difficult if not impossible to tell whether a filament is a nerve fiber or a neuroglia fiber, the difference between them being rather insignificant aside from the anastomoses, which, judging from the stained specimens, frequently occur between the neuroglia fibers but never between the nerve fibers. In silver-stained specimens, however, there is a marked

[2] Ingebrigtsen, R., *Jour. Exper. Med.*, 1913, xvii, 182.
[3] Ingebrigtsen, R., *Jour. Exper. Med.*, 1913, xviii, 412.

difference between the grown out nerve fibers and the neuroglia syncytium.

As shown in Fig. 12, the filaments supposed to be neuroglia are not quite cylindrical but are unevenly shaped; they are provided with varicosities, and between the latter the protoplasm is granular. The main point, however, is the presence of anastomoses between different fibers, resulting in a real framework-like syncytium in the plasma. The difficulties encountered in the fixation of the cultures have made my experiments aiming to a coloration by means of a method supposed to be more or less specific for neuroglia entirely unsuccessful, so aside from the silver-stained preparations I have studied them by means of basic hematoxylin. I cannot, therefore, prove that the fibers just described are neuroglia, but indirect evidence supporting this view can be produced by excluding what we know they are not. Previous investigations (Carrel and Burrows, Ingebrigtsen) have shown that connective tissue and endothelial tissue grow in plasma in a manner quite different from the structures under consideration. As demonstrated from the silver preparations the nerve fibers growing out from ganglia cells also display a different aspect. And then the only conceivable idea concerning their origin is the assumption that these fibers are growing neuroglia tissue.

Fig. 13 A is produced by a combination of three photographs of a culture 3 days old from a piece of cerebellum taken from a young cat. The photographs have been focused on three different planes, following the fibers in their course from their beginning in a big cell of the cortex of cerebellum out to their branches and ends in the plasma. Fig. 13 B is a drawing reproduced from the combined photographs, bringing out details of the preparation which have been omitted in the photographs. These illustrations show how the protoplasmic filaments growing out of the big cell branch, anastomose between themselves, and with other fibers from the neighborhood form a real syncytium built of protoplasmic filaments of different size.

DISCUSSION.

The Wallerian degeneration of a peripheral nerve means the death and disintegration of the axis cylinders and myelin sheath.

From the work of Ranvier[4] we know that the process is influenced in its rate by various agencies; thus it occurs more quickly in young animals than in older ones, in vigorous and healthy individuals than in the sick, more quickly also in warm-blooded than in cold-blooded animals, and in mammals it occurs more quickly in rabbits, for instance, than in dogs.

But in any case the various stages of degeneration follow each other in a typical way quite different from that of a nerve in the dead body.

Mönckeberg and Bethe[5] have observed that the first stages of the Wallerian degeneration may occur in a dead animal when it is kept at body temperature for some hours, but that it stops within the first twenty-four hours. I have confirmed the results of these investigators and found that there is no progress of the degeneration of the nerves after twelve hours in a dead body kept at 38°. As the death of the individual is different from cellular death, the latter occurring later than the former, Mönckeberg and Bethe concluded that the Wallerian degeneration is a process connected with living tissue.

Merzbacher[6] confirming this view added that the occurrence of a Wallerian degeneration in a nerve means that this nerve is in a condition of survival.

Nageotte[1] succeeded in producing the first stages of Wallerian degeneration in nerves kept in survival *in vitro*, and his work has demonstrated that the segmentation of the axis cylinders and myelin sheaths is independent of, and does not, as believed by Ranvier, result from the functions of the protoplasm of the cells of Schwann. The conception of Nageotte concerning the segmentation of the myelin-clad axis cylinders is that the myelin sheath, which is built of living protoplasm rich in mitochondria, digests the axis cylinders in the closed ovoid cavities formed by its segmentation, dying later after having performed this function. This conception of the part played by the myelin sheath in the destruction of the axis cylinder has been adopted by Cajal. The autodigestion of the myelin-clad

[4] Ranvier, L. A., Leçons sur l'histologie du système nerveux, Paris, 1878.
[5] Mönckeberg, G., and Bethe, A., *Arch. f. mikr. Anat.*, 1899, liv, 135.
[6] Merzbacher, *Neurol. Centralbl.*, 1905, xxiv, 150.

axis cylinders develops *in vitro* in a solution containing sodium chloride and salts of some bivalent metal in certain proportions. This observation of Nageotte demonstrates that the segmentation of the myelin sheath obeys the laws of Loeb as does the life of living animals (Nageotte). It obeys also the other laws of biology. It does not occur in nerves kept at 0° nor in those kept at 45° C.

I have found that the segmentation of the myelin sheath is completely abolished in plasma. This does not mean that the nerves kept in plasma are dead; for we know (Carrel and Burrows[7] and Ingebrigtsen[8]) that plasma is by far the best medium for the maintenance of life of tissue outside of the body, and we have a direct proof of the survival of nerves kept in plasma from the fact that in this medium the cells of Schwann give rise to a rich proliferation. And nerve fibers incubated in plasma for twenty-four and forty-eight hours develop a Wallerian degeneration when incubated afterwards in Ringer solution. The proper ratio of calcium and sodium salts is present in plasma as well as in serum, and the addition of calcium chloride did not produce segmentation.

It is difficult to understand how Wallerian degeneration is inhibited in plasma. The reasonable conclusion seems to be that the nerve does not die and degenerate in plasma, because it exists there in extremely favorable conditions, more favorable than those prevailing in the organism, where the peripheral part of a divided nerve must degenerate.

Proliferation of the cells of Schwann does not take place, however, even in plasma unless the first stages of the myelin segmentation have developed in the fibers already before their implantation in the new medium. While fibers removed from the peripheral part of a divided nerve within the first 4 days after section give rise to no growth in plasma, a growth occurred in 16 per cent of the cultures prepared from nerve fibers on the 5th day of Wallerian degeneration, and from later stages an increasing percentage of positive cultures was recorded.

We know that in the peripheral part of a divided nerve in rabbits a hypertrophy of the protoplasm of the cells of Schwann is visible on

[7] Carrel, A., and Burrows, M. T., *Jour. Exper. Med.*, 1911, xiv, 244.
[8] Ingebrigtsen, R., *Jour. Exper. Med.*, 1912, xvi, 421.

the 4th day; their multiplication begins about the 6th day, when the segmentation of the myelin sheath is well started. From these facts it may be concluded that the cells of Schwann are quiescent and unable to multiply up to a certain stage of myelin and axis cylinder segmentation, and that the latter and not the section of the nerve is the direct cause of their proliferation.

Our interpretation of the protoplasmic structures growing into the plasma from the cut ends of the nerve fibers seems to be conclusive; it is clear from the illustrations that they are direct outgrowths from the cells of Schwann, characterized by a tendency to form a syncytial framework. Is this an indication that such an outgrowth occurs from the cut end of a degenerating nerve (central end of peripheral segment) in the organism, and plays some part in the processes of union and regeneration? I have never seen it and I am not aware that it has been noticed by other observers. But it is probable that such a growth from the cells of Schwann of the peripheral part of a divided nerve extends into the scar tissue forming part of it. That structures of this kind have not yet been detected in the organism does not prove their non-existence. They stain faintly, and curving and bending through the scar tissue it may be difficult to bring out their true form.

The centrifugal orientation of the young axis cylinders growing out from the central part of a divided nerve has been explained by Forssman,[9] and his explanation has been accepted and modified by Cajal[10] on the assumption of positive chemotactic influences exerted by the cells of Schwann of the peripheral part. This assumption is merely hypothetical. An anatomical conception of the centrifugal orientation based upon my experiments seems more satisfactory, assuming that the protoplasmic syncytium of Schwann growing out from the peripheral part of a divided nerve, branching in the scar may receive the axis cylinders coming from the central part and serve as a guidance for them into the peripheral segment. In no case did I observe axis cylinders forming inside or growing out from the incubated nerve fibers. The fact that axis cylinders grow out into the plasma from pieces of the central nervous system containing ganglia

[9] Forssman, J., *Beitr. z. path. Anat. u. z. allg. Path.*, 1898, xxiv, 56; 1900, xxvii, 407.
[10] Cajal, Studien über Nerveregeneration, Leipzig, 1908.

cells, is an additional argument in favor of the monogenetic theory of the regeneration of nerves.

From a morphological standpoint the study of the grown out syncytium of Schwann in the plasma presents some features of interest. The study of the structure of the protoplasm of Schwann is a difficult task, and it is only in the last few years that the work of Nageotte has elucidated its syncytial nature. The protoplasm growing out from the cells of Schwann in the plasma in the first stages of the Wallerian degeneration shows a tendency to form a syncytial network. In the protoplasmic structures growing out in the later stages of Wallerian degeneration the liability to branch and anastomose is not so pronounced; the straight parallel rows of cells are similar to the so called bands of von Büngner.[11] The bands of von Büngner have been interpreted by this investigator and several others after him as the first signs of a differentiation of an axis cylinder out of the multiplied and coalesced cells of Schwann in a degenerating nerve. The errors of this interpretation, however, have been shown by Cajal[10] and Nageotte.[12] My photographs of the syncytium of Schwann growing in plasma, and showing the morphological likeness between the structures and axis cylinders, give an additional demonstration of the error. It is interesting to note the corresponding appearance of a syncytium of Schwann and syncytial neuroglia growing in plasma. There is very little difference between the pictures, and this is not surprising, when we take into consideration the common origin of these tissues in the early fetal life of the organism.

CONCLUSIONS.

1. The Wallerian degeneration occurring in peripheral nerves by incubation in Ringer solution and serum does not occur in plasma.

2. Peripheral nerves incubated in plasma give rise to no growth. The same is true of peripheral nerves in a Wallerian degeneration up to the 4th day.

3. Peripheral nerves in Wallerian degeneration from the 5th day give rise to a growth of the syncytium of Schwann. In cultures from

[11] von Büngner, O., *Beitr. z. path. Anat. u. z. allg. Path.*, 1891, x, 321.

[12] Nageotte, *Compt. rend. Soc. de biol.*, 1911, lxx, 861.

later stages there is a progressive growth of the same structure. It is evident that the proliferation of the cells of Schwann is directly produced by the degeneration of the axis cylinder and its myelin sheath.

4. In no case was growth of axis cylinders observed.

5. The growth of the syncytium of Schwann from degenerating nerves affords a basis for an anatomical conception of the centrifugal orientation of growing axis cylinders in regeneration.

6. Morphologically there is a striking resemblance between the syncytium of Schwann and neuroglia growing in plasma.

EXPLANATION OF PLATES.[13]

PLATE 34.

FIGS. 1 AND 2. Microphotographs of nerve fibers from the sciatic nerve of a rabbit, incubated in plasma for 12 days. Hardened in formalin and stained with hematoxylin. Fig. 1, low power magnification; Fig. 2, high power magnification. There is no trace of Wallerian degeneration.

FIG. 3. Microphotograph of protoplasmic filaments (syncytium of Schwann) emerging into the plasma, from the cut end of a nerve segment removed from the peripheral part of a divided nerve 5 days after section. Hardened after 6 days of incubation and stained with hematoxylin.

In the center of the picture a fiber is seen including a nucleus of Schwann and three myelin (fatty) globules. Several connective tissue cells are also seen.

PLATE 35.

FIG. 4. Camera lucida drawing of framework-like syncytium grown out from the cut end of a nerve segment removed from the peripheral part of a divided nerve, 5 days after section and incubated in plasma for 6 days. Stained with hematoxylin.

The nuclei of Schwann are surrounded by myelin globules.

PLATE 36.

FIG. 5. Camera lucida drawing of branching protoplasmic filaments grown in plasma, from the cut end of a nerve segment removed from the peripheral part of a divided nerve 6 days after section. Incubated for 5 days and stained with hematoxylin.

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[13] Dr. O. Berner, of the Anatomical Institute, Christiania, kindly made some of the microphotographs, Dr. Platou a few of the photographs.

PLATE 37.

FIG. 6. Microphotograph of protoplasmic structures grown in plasma, from the cut end of a nerve segment removed from the peripheral part of a divided nerve 6 days after section. Incubated for 9 days. Cajal silver impregnation. Low power magnification.

FIG. 7. Camera lucida drawing from the same preparation. The length of the fibers is about 350 microns.

Note the varicosities produced by nuclei.

PLATE 38.

FIGS. 8 AND 9. Microphotographs of protoplasmic structures grown in plasma, from the cut end of a nerve segment removed from the peripheral part of a divided nerve 13 days after section. Incubated for 5 days and stained with hematoxylin. Fig. 8, low power magnification; Fig. 9, high power magnification.

PLATE 39.

FIGS. 10 AND 11. Microphotographs of protoplasmic structures grown in plasma, from the cut end of a nerve segment removed from the peripheral part of a divided nerve 19 days after section. Incubated for 6 days and stained with hematoxylin. Fig. 10, low power magnification, showing the whole bunch of filaments; Fig. 11, high power magnification, giving details from the same preparation.

PLATE 40.

FIG. 12. Microphotograph of the branching and anastomosing syncytium grown in plasma, from a piece of cerebellum of a young dog. Culture 2 days old stained with hematoxylin. High power magnification (1,900 diameters).

PLATE 41.

FIG. 13. Microphotographs (A) focused on three different planes of branching and anastomosing syncytium grown out from a big neuroglia cell of a piece of cat cerebellum. Culture 3 days old. Cajal silver impregnation. B is a drawing of the same structures.

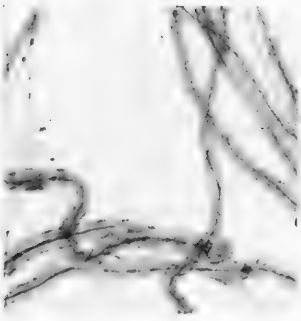

FIG. 1.

FIG. 2.

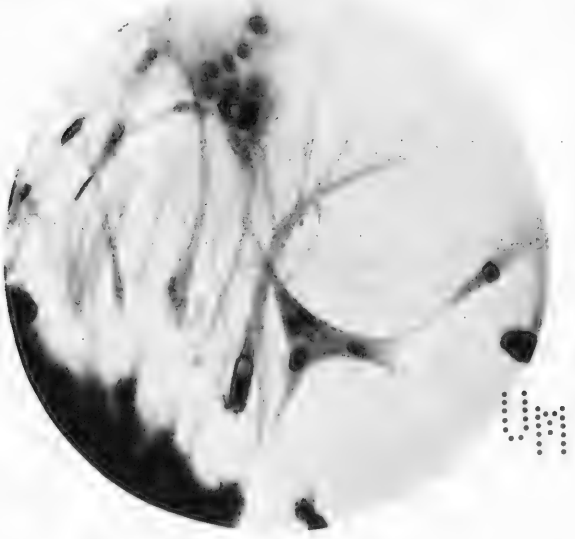

FIG. 3.

(Ingebrigtsen: Biology of Peripheral Nerves in Transplantation.)

FIG. 4.

(Ingebrigtsen: Biology of Peripheral Nerves in Transplantation.)

FIG. 5.
(Ingebrigtsen: Biology of Peripheral Nerves in Transplantation.)

FIG. 6.

FIG 7.

(Ingebrigtsen: Biology of Peripheral Nerves in Transplantation.)

FIG. 8.

FIG. 9.

(Ingebrigtsen: Biology of Peripheral Nerves in Transplantation.)

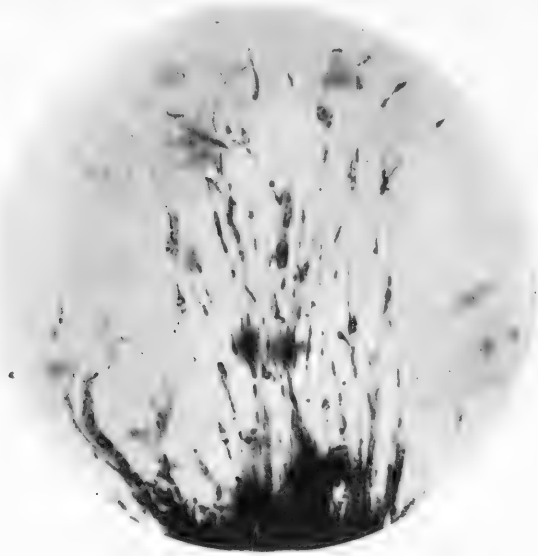

FIG. 10.

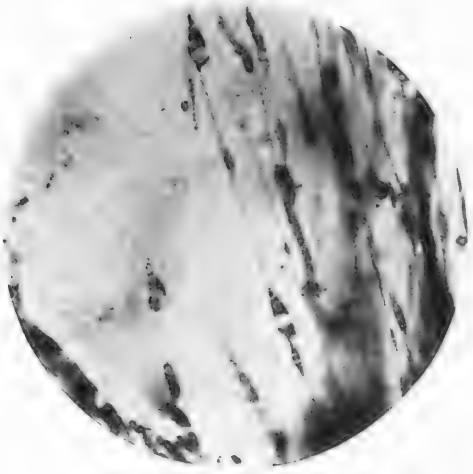

FIG. 11.

(Ingebrigtsen: Biology of Peripheral Nerves n Transplantation.)

FIG. 12.

(Ingebrigtsen: Biology of Peripheral Nerves in Transplantation.)

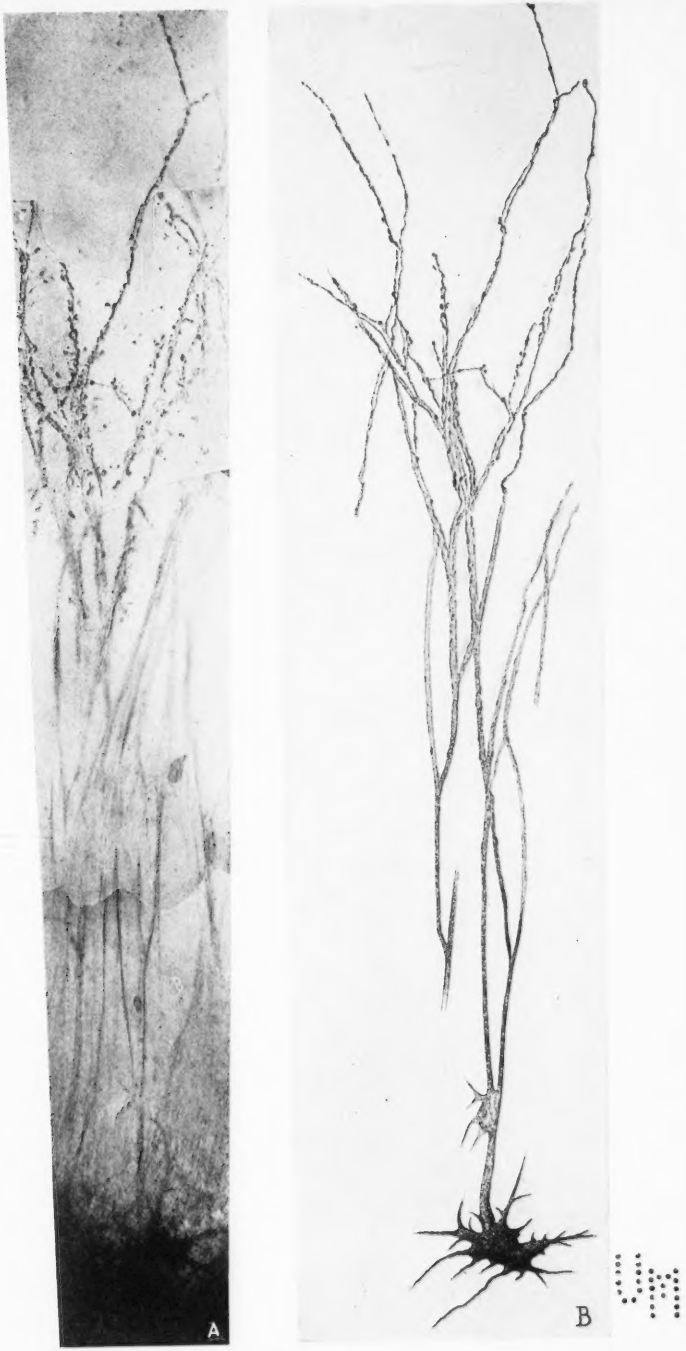

FIG. 13.

(Ingebrigtsen: Biology of Peripheral Nerves in Transplantation.)